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DESICCATION OF MICROORGANISMS AND BIOLOGICAL PREPARATIONS

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DESSICCATION OF MICROORGANISMS AND BIOLOGICAL PREPARATIONS

## INTRODUCTION

In carrying out measures to control infectious diseases in farm animals no small role is played by the improvement of methods for using and preserving commercial and laboratory bacterial cultures, filtrable viruses, and biological preparations. The necessity for developing new methods in this field is voiced by the demands of socialist animal husbandry.

The search for methods for the preservation over a long time of the biological properties of microorganisms and filtrable viruses as well as the active properties of biological preparations is of practical as well as theoretical importance.

The methods for preserving the biochemical and virulent properties of microorganisms in nutritive media are as yet far from perfect since the bacterial cultures at times radically change properties under the influence of the medium. The preservation of virulent properties of commercial bacterial cultures and filtrable viruses by passing them through the bodies of larger animals necessitates substantial material expenditures and even then this method preserves the virulent properties for comparatively short periods, measured in terms of several months.

At present there are many facts which confirm the variability of microorganisms when preserved in nutritive media. This can be explained by the fact that the nutritive media are not strictly constant in their physical and chemical properties. In addition changes in the media connected with metabolic processes of the microbes have an effect on the biological properties of the latter.

In a like degree the biological properties of filtrable viruses also depend on the surrounding medium. For this reason in working with cultures of bacteria and filtrable viruses at research institutes and plants producing biological products the most important problems concern the preservation of the original biological properties.

The preservation of biological preparations in a liquid form (living bacterial vaccines, certain immune sera, and penicillin) creates a number of difficulties connected with the fact that these preparations soon lose their active properties. For this reason we thought it necessary to give a description of current methods employed in the production, use, and preservation of dry cultures, viruses, and biological preparations.

The most effective method, as shown by the experience of recent years, is that of drying. The drying of bacterial cultures, filtrable viruses, and biological preparations has become widespread in medical practice especially since the proposal of new principles regarding drying and the apparatus. In veterinary medicine however the technology for producing dry biological preparations has hardly been developed and the theoretical questions involved in drying cultures of microorganisms and filtrable viruses and in their preservation in a dry form have hardly been studied. In this connection it was thought to be timely, from our point of view, to start on the search for better methods for the preservation of bacterial cultures, filtrable viruses, and biological preparations prepared for veterinary practice.

In this book, along with a survey of monographic studies of the problems of drying biological preparations, cultures, and viruses, we have given considerable attention to our own research in this field since these problems have not yet been satisfactorily solved.

In our research we started from materialist Michurinist principles and methods since only an active intervention in the life processes of the bacterial cultures, through varying the conditions surrounding the microorganisms and biological preparations, could be useful and scientifically correct in solving the problems before us.

Since the state of protein substances depends on the surrounding environment, in order to solve the problems of preserving the active properties of bacterial cultures, filtrable viruses, and biological preparations over a long period of time it was necessary to create a new environment by drying and storing under vacuum.

In carrying out our research in this field we aimed at accomplishing the following: (1) develop methods and improve apparatus for drying microorganisms, filtrable viruses, and biological preparations with the aim of preserving them for a long time; and (2) study the biological properties of the dry bacterial cultures, filtrable viruses, and biological preparations during storage.

The methods which we developed for drying in a vacuum from a frozen state make it possible to preserve bacterial cultures, filtrable viruses, and biological preparations for a very long time.

The use of these drying methods permits the microbiologist to foresee the behavior of the bacterial cultures and viruses when setting up special experiments. In addition these methods account for a marked economy of material which is usually expended in the preservation of bacterial cultures and viruses by the usual method of passing them through inoculated larger animals. The method of

drying bacterial cultures and filtrable viruses has great significance for the biological industry since the strains needed for work in immunology can be kept in a dry state for a very long time.

The use in practice of dry vaccines and serum preparations and penicillin permits us to carry out control measures against infectious diseases of farm animals more successfully.

#### CHAPTER I. HISTORICAL SURVEY

In microbiological practice attention has long been given to the problem of producing dry biological preparations and bacterial cultures. For example, the method of drying antitoxic sera for purposes of standardization has been in existence since the end of the past century. The possibility of preserving toxins and antitoxins in a dry state has permitted their use in practice as international standards even up to the present time.

The drying method has special significance for the long term preservation of virulent properties of microorganisms and filtrable viruses, as well as serum preparations and penicillin, all used for preventive inoculations and therapeutic and diagnostic purposes.

Sometimes it is difficult to preserve such typical features of microorganisms as their toxic and virulent properties and the biochemistry of their cultures. For instance, the ordinary method for prolonged preservation of strains on nutritive media made necessary frequent reseedings. The latter not infrequently leads to a change in the standard features of the strains and their virulent properties in particular. As for the preservation of filtrable viruses, here we have even more difficulties in view of the slight resistance, as a rule, of the viruses to the activity of the surrounding medium.

Attempts of several investigators to develop a method for drying microorganisms and biological preparations in desiccators over chemical absorbents or in heated drying chambers did not give positive results.

Shackell (1909) made a report on a new principle for drying from a frozen state in a vacuum. The author used this method in drying fixed rabies virus and guinea pig complement. However Shackell's experiments did not attract the attention of researchers for the reason that they were of an unsystematic nature.

In the years following, many investigators made experiments on drying bacterial cultures, filtrable viruses, and biological preparations with contradictory results.

#### Bacterial Cultures

In 1916 I. L. Serbinov first pointed out the great resistance of microorganisms to drying. According to his data, cultures of Azotobacter preserved their viability for 160 days following drying in a desiccator. The author likewise reported on the extraordinary resistance to drying of the Lactobacilli.

Later there periodically appeared in the scientific literature isolated communications on drying bacterial cultures. However this method was not used for a long time for the prolonged preservation of bacterial cultures.

After 1934 medical research institutions started to work out methods for drying bacterial cultures, filtrable viruses, and biological preparations and soon positive results were obtained in this field.

In 1942 A. Klimentova, R. Kurk, and G. Yarmol'chuk (All-Union Institute of Experimental Medicine) made a report on results of drying cultures of *Corynebacterium diphtheria* and Shiga's and Flexner's bacilli. During the initial stage of their experiments these authors dried these cultures in a vacuum desiccator over calcium chloride and then in a manifold-type apparatus. By testing the dry cultures obtained it was established that the diphtheria bacteria preserve their cultural biochemical and virulent properties for 4 years while Shiga's and Flexner's bacilli preserve their cultural, toxigenic, and agglutinogenic properties for 6 years.

A. R. Konova and L. S. Bazilevskaya used a culture drying method for the preservation of their biological properties. In a work published in 1938 the authors reported on drying bacterial cultures in a desiccator over calcium chloride in a vacuum without preliminary freezing. The drying process in each individual case lasted 2-3 days. In testing the toxicity of dry diphtherial strains it turned out that it was more pronounced in the dry than in the original cultures.

In 1940 N. A. Kazberyuk reported on the successful drying and preservation of bacterial cultures of Shiga, Flexner, and Hiss dysentery and paratyphoid fever. He dried the cultures in a frozen state in a vacuum desiccator for 2-3 days.

In 1941 S. S. Rechmenskiy successfully used a method for drying microorganisms from a frozen state in a vacuum apparatus. In his experiments Rechmenskiy used a saprophytic strain of *Vibrio aquatilis* and a culture of *Escherichia coli* for the purpose of preserving their biological properties for a long time.

In 1940 N. S. Iitter described his experiments in drying streptococcal cultures from a frozen state in a vacuum desiccator. The dried cultures were kept in test tubes wither sealed with paraffin or fused shut. When 16 dried strains were tested it was found that 5 strains preserved viability for from 3 to 5 months, 7 strains from 6 to 8 months, and 4 strains from 9 to 11 months.

K. Ye. Dolinov and L. B. Balayan in 1946 described a method for drying pneumococci from a frozen state in a high vacuum. As a protective medium during drying the authors used a nutritive broth and serum, the latter being the more effective. By using this method, the authors point out, it is possible to preserve permanent cultural biological properties of pneumonococci for 38 months.

N. N. Titov (1948) dried staphylococci, Eberthella typhosa, and Mycobacterium tuberculosis from a frozen state on filter paper. The dried microorganisms, sealed in pipettes under a vacuum, kept their viability for more than a year.

In 1948 Yu. A. Kozlov, B. S. Del'nik and V. V. Vinogradov reported on the use of method for preserving museum cultures in a dry state. The authors dried cultures of Eberthella typhosa, A and B paratyphoid bacilli, Brucella, staphylococci, Escherichia coli, etc. The frozen cultures were dried for 6 hours in a vacuum. As a drying medium the authors used a 10% solution of saccharose and 1.5% gelatine. As a result of their observations of the dried cultures for a year it has been established that many labile cultures may be preserved by the drying method.

Foreign scientists have likewise been occupied with the search for methods for long term preservation of biological preparations and bacterial and virus cultures.

In 1935 a report was published on experiments carried out in drying meningococcal cultures from a frozen state under vacuum. Years of observations showed that dry meningococcal cultures in a vacuum preserve their cultural biological properties for a very long time and may serve as standard samples in the work of research and educational institutes.

Cultures of meningococci, dried in a high vacuum refrigerator at a temperature of 40° for a period of 48 hours, standing in a desiccator at room temperature for 3 hours, and then stored in sealed test tubes without vacuum preserved viability for 89 days in the case of 2 strains and for 151 days in the case of 4 strains. In this instance the cultures could not be kept successfully in a dried state which would indicate incomplete drying.

Cultures of pneumococci, dried in a mixture with rabbit blood by an open method at room temperature preserved their viability for from 2 to 13 months. The strains of avirulent pneumococci (rough-type colonies) retained viability much longer than the virulent ones (smooth colonies). Such a short period of preservation of viability in dry cultures must be explained by the fact that the cultures were stored without vacuum as well as by the fact that the drying method used was not very effective.

A study of the effect of the medium on the results of drying cultures of *Streptococcus pyogenes* and *Escherichia coli* indicated that colloids (starch, gastric juice, and peptone) used in the form of 1% solutions have more pronounced protective properties than solutions of crystalloids (sodium chloride, tryptophan, xylose, glucose, and sucrose). The best results are obtained from drying media consisting of a mixture of colloid and crystalloid solutions.

Positive data on drying and preserving viability and other properties of dried bacterial cultures, spoken of before, were obtained by Klimentova, Kruk, Yarmol'chuk, Dolinov and Balayan, Kolesov, Rechmenskiy, etc. The success of Soviet scientists can be explained by the fact that in their work they have been guided by the basic tenets of Michurinist biology, the doctrine of the decisive influence of the surrounding medium on the development of the living organism. In addition, their research has had a systematic character and has been carried out with a large number of species of microorganisms.

#### Bacterial Vaccines

Of special importance for medical and veterinary practice is the drying of living bacterial vaccines which makes it possible to preserve the immunogenic properties of these preparations for a long time.

K. Ye. Dolinov has for a number of years been preparing a dry BCG vaccine, using the method of drying in a manifold vacuum apparatus from a frozen state. For a drying medium the author has used a glucose solution. The BCG vaccine, when dried by this method, retained its immunogenic properties for a year.

D. F. Fedorov, A. A. Batyрева and O. N. Kocher'yan reported in an article published in 1944 on experiments which they had done on producing a dry BCG vaccine in a vacuum apparatus at low temperatures. In drying the vaccine the authors used 25% normal horse serum, Calmette's fluid, a physiological saline solution and mother's milk as protective media. The best results were obtained using serum. In testing the dry BCG vaccine it was found that it retains its viability and active properties much longer than the liquid vaccine.

L. I. Nakhimson and F. M. Neymark (1948) reported on positive results in drying BCG vaccine from a frozen state in a vacuum with 10% sucrose and 1% gelatin for a drying medium. The authors indicate that this medium assures the preservation in the dry vaccine of up to 20% of the living bacterial cells.

Yu. A. Kozlov in 1948 published data from his experiments in freeze-drying BCG vaccine in a vacuum. For a drying medium he added to the vaccine 10% sucrose and 1.5% gelatin, or 10% sucrose, 1.5% gelatin, and 0.1% agar. The author points out the advantages of the drying media which he used over glucose as regards better preservation of the living bacterial cells in the vaccine.

A. A. Dorofeyev and V. M. Putimov in 1945 proposed a method for preparing a dry brucellosis vaccine for inoculating cattle from strain 19. The dry vaccine when tested on laboratory animals and cattle was stable and suitable for use in the field.

M. M. Faybich in 1946 reported on the preparation of a dry living sucrose-gelatin plague vaccine from the E. V. Girard and Robiquat strain by drying it in a vacuum. The dry plague vaccine thus obtained differed radically from the liquid vaccine since, when kept in a refrigerator (2-4°), it retained potency 36 times longer than did the liquid vaccine. The great stability of the bacteria in the living plague vaccine can be explained by the fact that the vaccine was freeze-dried in a high vacuum and a 10% sucrose solution and 1.25% gelatin was used as a protective medium.

M. M. Faybich and T. S. Tamarina (1946) worked out a method for making a dry living tularemia vaccine by vacuum freeze-drying. The authors made the vaccine from the attenuated strains Gaysk muskrat IV and No 15, using a 10% sucrose solution and 1.25% gelatin

for a drying medium. Tests of the preparation thus obtained on white mice and guinea pigs showed that it retains its immunogenic properties for 1.5 years when stored under vacuum in vials at a room air temperature of  $2-4^{\circ}$ . The results of using dry vaccine in medical practice has demonstrated its great advantages over the liquid form by the fact that it remains potent for 1.5 years instead of 12-15 days.

#### Filtrable Viruses and Virus Vaccines

T. Ya. Vannovskiy (1932-1933) reported on the experiments which he had carried out on drying the sheep pox virus in a vacuum desiccator over sulfuric acid. When testing the dry sheep pox virus for virulence he established that within 3.5-9 and even 14 months after drying the virus was capable of inducing a typical local reaction when sheep were inoculated. The virus was avirulent when tested after 18 months.

In veterinary practice dry vaccine is being used to control fowl pox (dove pox virus). The preparation method consists of drying a liquid vaccine in a vacuum desiccator over calcium chloride. The dry mass is pulverized in a mortar. This vaccine however has a limited keeping time (3 months) due to imperfections in the preparation method used.

K. N. Pochechuyev in 1936 reported on the preparation of thermostable smallpox calf lymph by drying the virus in a thermostat at  $37^{\circ}$  for 9-10 days. For a protective medium when drying the virus the author used gelatin, eggwhite, horse serum, gum arabic, and ox bile. When tested after 9 months of storage at room temperatures reaching  $43.5^{\circ}$ , the smallpox calf lymph, dried in gelatin, preserved virus activity better than that dried in any other medium.

A. A. Belyayev (1941), by studying the effectiveness of dry smallpox vaccine, established the possibility of preserving its activity for a long period by drying. The vaccine was freeze-dried in a vacuum. As a drying medium the author used lactose or eggwhite. The vaccine dried in lactose was more effective, according to Belyayev's data.

Other results were obtained by M. A. Morozov, M. I. Korol'kova, S. S. Kasatkevich, and K. Ye. Dolinov (1941-1942) in the preparation of dry smallpox vaccine by freeze-drying in a vacuum apparatus. For a drying medium the authors tested sucrose, eggwhite, and a physiological solution, with eggwhite being the best medium for preparing dry vaccine. According to the data of these authors the activity of dry smallpox vaccine was retained for 18 months.

A. Klimentova, R. Kruk and G. Yarmol'chuk dried the virus of European relapsing fever. The virus thus dried retained its properties for 21 months.

D. A. Tsuverskalov (1944) made a dry ovinia preparation for vaccinating sheep against smallpox. The virus was dried under the direct action of calcined alumina over dry potassium hydrate in a vacuum. This dry ovinia preparation retained its active properties for 10 months at temperatures reaching 30-32°.

In the Institute of Neurology of the Academy of Medical Sciences (section of neurotropic virus infections) headed by academician M. P. Chumakov) a method for drying filtrable viruses from a frozen state in a vacuum apparatus has been used since 1941. The causative agents of human and animal encephalitis, Omsk and Crimean hemorrhagic fevers, influenza and choriomeningitis viruses, etc have been dried. According to M. P. Chumakov's data, this method made it possible to preserve under vacuum all the viruses indicated and to use them in experimental work.

In 1939 a method was described for drying tissues from malignant tumors in poultry caused by filtrable viruses (Rous sarcoma, Fujinami microsarcma). The dried tumor tissues were tested soon after preparation and after being preserved for 10 months in a vacuum or in dry nitrogen. As a result of these tests it was established that the drying process for tumor tissues did not have a harmful effect on the virulence of the filtrable viruses. The dry viruses obtained by this method caused a typical disease and death in the expected period when poultry were inoculated.

In 1942, thanks to the drying method, it was made possible to keep the distemper virus for a long time. In a frozen state splenic pulp from a dog with distemper was dried in a vacuum apparatus and kept in vacuum vials or covered with dry nitrogen. When tested for virulence on ferrets 430 days after drying the dry virus caused the disease and the death of the animals in the established time limits.

#### Therapeutic Sera and Penicillin

In veterinary practice in the USSR N. N. Levitov and D. A. Tsuverkalov were the first to pose the question of the preparation of a dry vaccine against malignant anthrax for use in veterinary and medical practice. They obtained a concentrated anthrax serum by using acetone to precipitate protein fractions containing antibodies and then drying them with ether. When tested for activity in rabbits the dry anthrax serum had pronounced immune properties. However this method did not find acceptance in practice.

In 1940 the work of G. Ya. Rozenberg was published, dealing with the production of dry human blood plasma and serum. In 1944 G. Ya. Rozenberg reported on an apparatus which he had designed

for drying purposes and the results obtained in producing and studying dry plasma and serum. The plasma was dried for 5-7 hours in a vacuum without preparatory freezing. In order to obtain total solubility the author added glucose to the original preparations. Tests of dry plasma and serum a year old showed them to be highly effective for human and rabbit transfusions in cases of great blood loss. When testing for physical and chemical properties the author established that the dry preparations obtained were completely soluble and retained all the physical and chemical factors which had been determined before drying.

In 1943 L. G. Bogomolova reported on the production of dry plasma and serum and their use in clinical practice. According to Bogomolova's data, the drying method was perfect, guaranteeing the preservation of all qualitative properties of the plasma and serum for 2 years. When the author tested the dry preparations for physical and chemical properties she did not find any substantial deviations from the original. Experiments in using dry plasma and serum in animals and human beings showed that the dry plasma and serum have pronounced hemostatic and blood substitute functions in loss of blood, dystrophy, and other diseases.

Positive results in the production of dry anti-measles sera were obtained by L. I. Kolesnikova and M. V. Kholchev (1939-1943). They dried frozen sera in a vacuum apparatus. The dry sera were easily dissolved in a physiological solution and were effective for a period of 2 years.

In 1945 N. M. Titov published data from his experiments in drying biological preparations. The first experiments in drying some therapeutic veterinary sera were carried out with a spray-disk

apparatus in air heated to  $110^{\circ}$ . However when tested at the State Control Institute of Veterinary Biological Products the sera dried by Titov were contaminated with foreign microflora. The author later used an improved method for drying the medical biological preparations from a frozen state in a vacuum chamber and positive results were obtained.

In 1935 a new method was suggested for preparing dry convalescent sera against scarlet fever and measles and other biological preparations. These sera were freeze-dried under high vacuum. The dry convalescent sera gave positive results when used in a concentrated form against scarlet fever and measles.

A number of works reported on the extensive use of a method for drying human blood serum and plasma for therapeutic purposes as a blood substitute in cases of blood loss or other diseases. The drying was done from a frozen state in a specially constructed vacuum apparatus. After drying the flasks with the serum and plasma were filled with dry nitrogen and kept sealed until used.

It is also necessary to point out the extensive use of penicillin therapy in medical practice. Recently penicillin has been showing up in veterinary practice for treating strangles, swine erysipelas, and certain other diseases. Penicillin cannot be used in practice in a liquid form as a therapeutic agent since it is unstable and can be kept for only a few days. In view of this fact, penicillin is used only in a dry form. Dry penicillin keeps its therapeutic properties for a very long time (for a year or more). In the Soviet Union effective methods have been developed for drying penicillin which are much superior to those in use in foreign countries. It must be pointed out that the success of penicillin therapy depended to a great extent and still depends on the method for freeze-drying penicillin in powerful vacuum chamber apparatus.

### Diagnostic Preparations

In 1939 I. S. Avessalomov reported on the preparation of a dry trypanosomic antigen for use in the complement fixation test. The drying was done in Petri dishes, kept for several days at 37°. The dry trypanosomic antigen was active and usable for several years. M. I. Fedorovich (1942) for a long time used dry antigen diagnostica prepared from typhoid and paratyphoid A and B micro-organisms for the agglutination test. The author dried the antigens in a hot-air chamber at 37°, in an apparatus with air-water heat at 40-45° and in a desiccator over calcium chloride. The dry antigens retained pronounced agglutinative properties for 34 months.

From the reports of Baglikova on the production of cultures for diagnostic purposes by drying on filter paper in a vacuum at room temperature it is apparent that a dry phenolized culture of *Proteus* X-19 retained its activity for only 3 months. Attempts to use this same method in preparing typhoid and paratyphoid cultures for diagnostic purposes were unsuccessful. A. M. Poltinnikova (1942) obtained dry cultures for diagnostic purposes and bacterial preparations by various drying methods, including in a hot-air chamber at 37°, acetone and ether, alcohol and ether, in a vacuum apparatus at -78° and in an "anaerostat." Of all these methods only that of the vacuum apparatus at -78° was reliable for the production of high grade preparations. When cultures of typhus bacteria and Flexner's and typhoid fever vaccine, freeze-dried in a vacuum, were tested, A. M. Poltinnikova established that dry vaccine and cultures for diagnostic purposes had the same qualitative properties as the originals.

A. Klimentova, R. Kruk, and G. Yarmol'chuk found that the dry agglutinative sera of Shiga's and Flexner's cultures, and paratyphoid B and typhoid fever retained completely their activity titer when tested 2 years after drying. In the laboratory of the Central Institute of Epidemiology and Microbiology K. Ye. Dolinov successfully prepares dry agglutinative sera for practice in large quantities. Laboratory practice has shown that dry sera retain their active properties for a very long time (5-6 years).

Dry serum from guinea pigs is being used for the complement fixation test in veterinary and medical practice. However there are a number of conflicting reports on this question. For example, Brin and Kolesinskaya indicate that dry complement is good for 30 months for use in the complement fixation test. B. I. Kurochkin reported, after making a comparative test of several series of dry complement, that after being sealed for 4 months in a flask with a rubber stopper at room temperature the complement loses 25% of its hemolytic strength, after 7 months 60%, and after 10 months 85%. S. S. Rechmenskiy, Ye. L. Gorokhova, and others found it possible to make prolonged use of guinea pig complement freeze-dried in a vacuum apparatus and stored under vacuum.

It can be considered unquestioned that the activity of complement may be stabilized for a long time only by freeze-drying under vacuum and storing the dried complement under vacuum conditions. The divergent results of testing the dry complement must be explained by the fact that the drying methods were apparently not very refined and the complement was stored without a low vacuum.

### Bacteriophages

There is a contradictory nature in the reports on the preparation of dry monovalent and polyvalent bacteriophages for certain infectious diseases. The method proposed by F. Ye. Sergiyenko in 1943 for drying the dysentery bacteriophage consists of drying the bacteriophage in a mixture with starch and normal horse serum in a hot-air chamber at 37° with calcium chloride as a moisture absorbent.

It was later established however that the pH of the bacteriophage dried in tablet form drops to 5.2-5.4, as a consequence of which its activity likewise drops. For this reason the bacteriophage was slightly alkalized before drying.

The drop in pH of dry bacteriophage during storage can apparently be explained by the large amount of residual moisture in it (10-12% after drying in a hot-air chamber).

B. I. Gol'dshteyn, G. M. Frenkel', and M. B. Gintsburg (1944) developed a method for making a dry dysentery bacteriophage by adding 600 g of ammonium sulfate to one lit of original bacteriophage. The authors took off the film which formed at the top, pressed out the liquid and mixed it in a mortar with 7.5 g of calcium carbonate and 10.25 g of anhydrous sodium sulfate until it formed a free-flowing dry mass from which the tablets were then formed. The dry bacteriophage thus prepared was usable for 11 months.

A. Ye. Kriss (1943) produced a dry dysentery phage vaccine from concentrated bacteriophage and dysentery vaccine. The dry dysentery phage vaccine, according to the author, had pronounced immunogenic properties.

V. V. Avrekh, A. N. Kalugina, and others in 1946 published the results of a comparative test which they made of the active properties of dry and liquid bacteriophages.

The authors indicate that the dry bacteriophage prepared by F. Ye. Sergiyenko's method was, as a rule, of slightly active lytic, antigenic, and agglutinative properties.

A. I. Gorokhovnikova established that dry dysentery bacteriophage, prepared by F. Ye. Sergiyenko's method, retains its activity for only 4-6 months instead of the 11 months obtained by the author who proposed the preparation. M. M. Priselkov (1948) tested the activity of dry dysentery bacteriophage prepared by F. Ye. Sergiyenko's method in parallel experiments with liquid bacteriophage and found that the dry preparation has less pronounced activity. Approximately the same results were obtained in experiments carried out by S. B. Dubrovinskiy, V. D. Rozenfel'd, and Z. A. Roshchina (1948).

## CHAPTER II. DRYING APPARATUS

### Desiccators and Drying Cabinets

For a long time it has been necessary to use desiccators and drying cabinets of various designs in drying biological preparations.

Vacuum desiccators have ordinarily been used for drying small quantities of antitoxins, toxins, and certain labile bacterial cultures. The drying process in these desiccators was carried out under vacuum. For absorbing moisture, use was made of granulated  $\text{CaCl}_2$ ,  $\text{KOH}$ ,  $\text{P}_2\text{O}_5$ , and other chemical absorbents which effect preliminary desiccation to a constant weight at  $180^\circ$ .

The material to be dried in test tubes or cylindrical vessels is placed in a desiccator over a moisture absorbent and the lid is closed tightly with vacuum grease. Then the air is pumped out of the desiccator with an oil vacuum pump. Air is pumped out periodically as long as the drying process lasts in order to maintain the vacuum at a specific level.

When the drying is taking place in large desiccators with sulfuric acid as the absorbent, a high vacuum (up to 1 mm in the mercury column) is necessary to accelerate the dehydration process. In order to increase the absorptive capacity of the sulfuric acid, it should be shaken up slightly. Otherwise its surface layers quickly become saturated with moisture. In order to speed up the process of drying certain serum and thermostabile vaccine preparations, they are placed in simple drying cabinets, drying cabinets with fans, vacuum chambers, and vacuum "anaerostats."

As a rule, in simple or vacuum drying cabinets drying takes place at 38-40°. The cabinets are heated electrically or with gas. In some cases the drying process is accelerated not only by heating but by the use of chemical moisture absorbents ( $\text{CaCl}_2$ ,  $\text{KOH}$ ) which are placed in the drying cabinets along with the material for desiccation. For instance, Fornet's drying cabinet has a device in the form of sensitive beam balances. The balance is connected with the dish in which the material is placed to be dried. The degree of desiccation can be determined by observing the pointer of the balance.

In the vacuum anaerostat the material to be dried is placed on specially adapted shelves. In order to speed up the drying process a chemical absorbent may also be placed in the anaerostat.

In addition, the vacuum anserostat has auxiliary hot-water heating for this purpose. The water fills the hollow space between the walls of the apparatus and is heated by an electric element (Figure 1).

At one time a drying apparatus with air and water heat was widely used (Figure 2). The water, which filled the hollow space between the walls of the apparatus, and the air are both heated by electric elements to 30-40°. The hot air is forced into the chamber by a fan. Drying is considered finished after the material has been dehydrated to a satisfactory degree and it can easily be ground to a powder.

The technology involved in drying is the same for all apparatus of the cabinet type. The open drying method is also used. In this case the material is poured in a shallow layer into Petri dishes or shallow pans, placed in the chamber and dried until a solid state has been reached. The preparation thus prepared is ground to a powder in a mortar or in a special mill and kept in tightly closed bottles.

The amount of residual moisture in preparations dried by this method frequently varies since it is very difficult to maintain constant technological conditions in this method. In view of this, the quality of biological preparations dried in these cabinets was likewise not always satisfactory. A great defect of the drying cabinets is the impossibility of obtaining a sterile preparation as well as the length of the drying process. In addition, as has been established, the preparations which contain animal protein lose certain physical, chemical, and biological properties during cabinet drying as a consequence of the denaturation of the protein. The loss of these properties diminishes the solubility and activity of the dry preparations.

### Spraying Apparatus

Many biological substances are dried in large apparatus by spraying in hot air. The defect of these apparatus is the fact that a sterile product cannot be obtained in them.

The problem of the preparation of a spray apparatus for drying human plasma and serum was successfully solved by G. Ya. Rozenberg. The author designed a spray apparatus (Figure 3) in which dry sterile plasma and serum could be produced in 5-7 hours. Drying in a spray apparatus takes place under a high vacuum which causes the formation of foam on the plasma and serum entering the drying ampule. The sprayed plasma condenses in a fine layer on the inner walls of the ampule and dries very rapidly. In this apparatus the water vapors from desiccation are absorbed by a refrigerated condenser located in a special bath with a cooling mixture (refrigerant). In order to speed up the drying process for serum the drying ampule of the spray apparatus is heated in a water bath at a temperature of 37-40°.

The spray apparatus developed by G. Ya. Rozenberg is thoroughly effective in drying a small quantity of human plasma and serum. It has also given a good account of itself in the preparation and use of these preparations in field and clinical practice.

### Manifold Vacuum Apparatus

The advantages of preliminary freezing of bacterial cultures and biological preparations in drying was confirmed by some investigators who did research in this field. It was established that the

freezing of immune sera and other biological substances eliminates the denaturation of protein and the loss of certain of their physical and chemical properties observed in the simple drying method. Nevertheless the method of drying biological substances from the liquid state in drying cabinets and desiccators has been employed for a long time.

The method of drying from a frozen state was first used to preserve labile microorganisms and certain diagnostic preparations. In 1921 a method was described for producing dry bacterial cultures. The cultures of microorganisms subject to drying were poured into test tubes which were placed in a desiccator, following prefreezing in a cooling mixture (ice with salt). The bottom of the desiccator was covered with glycerin as a conductor of cold while phosphoric anhydride was placed in a copper dish inside the desiccator to serve as a moisture absorbent. The lid of the desiccator was closed tightly by rubbing it well with a vacuum grease and the desiccator was placed in a bath with a cooling mixture. An oil vacuum pump was used to extract air from the desiccator and, with a vacuum of 2-3 mm on the mercury column, the whole apparatus was placed in a refrigerator where it remained until the drying process was finished. Test tubes with the dried bacterial cultures were filled with paraffin or were sealed shut.

A. Klimentova, R. Kruk, and G. Yarmol'chuk constructed and used a vacuum device for drying bacterial cultures and sera (Figure 4). Experiments employing this apparatus in drying bacterial cultures and sera were performed in 1934 at the All-Union Institute of Experimental Medicine. The cultures and sera were dried in a frozen state under a vacuum of 3 mm on the mercury column, created by using an oil vacuum pump. Calcium chloride or phosphorus anhydride were used as moisture absorbents. Ampules with the dried cultures were vacuum-sealed by fire.

The method for drying in desiccators was no longer used.

This can be explained by the insufficient production of the desiccators and the absence of the opportunity to employ constant cooling conditions and to create a high vacuum.

In 1935 an apparatus was proposed for preparing dry human convalescent serum (Figure 5). The basis of this method was the principle of preliminary freezing of the preparation and further drying in this state under a high vacuum. For condensing water vapors the authors used a refrigerated condenser cooled by dry ice and methyl "Cellosolve" at a temperature of  $-75^{\circ}$ .

The drying process in this apparatus was called lyophilic. The term "lyophilic" characterizes the original and undissipated capacity of the dried material for solution which indicates its unimpaired colloid chemical structure.

In 1937 Vzorov, Lang, and Kocher'yan made the LMO RIEM-4 vacuum apparatus with radially located ampules around a chamber. The moisture evaporated from the ampules containing the material for drying is absorbed in this apparatus by calcium sulfate placed in the chamber of the apparatus.

The material for drying is frozen in advance by dry ice and alcohol at a temperature of  $-75^{\circ}$ . Drying takes place under a high vacuum. The authors believe that this apparatus is perfect and assures the production of high grade dry bacterial cultures and biological preparations. For instance, they achieved positive results in drying certain bacterial cultures and BCG vaccine.

The advantage of the LMO RIEM-4 apparatus is the fact that the ampules with the material to be dried can be attached radially to the chamber with the absorbent and thanks to this the drying process can be greatly speeded up.

Comparing the principles of absorption and condensation of water vapor in the lyophilic process and in the method described in 1937 by Vzrov, Lang, and Kocher'yan, it must be pointed out that the method proposed by the Soviet authors is the better.

The method for condensing the water vapors in a refrigerator is more complex and expensive since for this a very low temperature must be provided ( $-75^{\circ}$ ,  $-78^{\circ}$ ) by dry ice and alcohol. On the other hand, the absorption of water vapors by calcium sulfate does not require much material and assures good drying of bacterial cultures, diagnostic agents, and certain preparations.

In 1938 the so-called cryochemical method for drying in a manifold apparatus was described (Figure 6). This method is distinguished from the lyophilic process by the fact that during drying the water vapor is absorbed by a chemical absorbent, calcium sulfate. As a consequence of this apparatus, the drying process is likewise distinguished from the preceding ones only by the fact that the manifold is connected directly with the chamber containing the calcium sulfate. Otherwise, everything including prefreezing and other processes is identical with the previously proposed method.

The construction of the radial manifold apparatus (Figure 7) is characterized by the fact that in it the manifold has radial lateral branches and is located in the form of a flange above the main condenser. The main condenser is a flasklike vessel inserted into a hollow metal tank containing a cooling agent (dry ice and alcohol) for cooling the condenser at a temperature of  $-78^{\circ}$ . Into the condenser is inserted a glass tube connected with a pipe passing to the vacuum pump through which air is extracted so as to

create a high vacuum throughout the entire apparatus. The hermetic nature of the apparatus is provided by metal flanges and packing at spots where the various parts join.

Water vapor passing from the radial ampules containing the material to be dried condenses and is converted into ice on the inner walls of the condenser. In addition, the apparatus has an auxiliary condenser ("trap") containing the agent mentioned above for condensing traces of moisture which may not be caught by the main condenser.

The degree of vacuum in the apparatus is determined by using a vacuum device and reaches, as a rule, 0.005 mm on the mercury column. The drying process lasts 30 hours after which the flasks with the material are vacuum-sealed and stored in a refrigerator. Despite the positive evaluation given by investigators this apparatus has not been widely accepted.

S. S. Rechmenskiy in 1941 described a method and device for drying biological preparations and microorganisms. His vacuum apparatus consists of a Langmuir mercury pump, a manifold, a vessel for drying the cultures, a condenser placed in the vessel with the cooling agent, a mercury vacuum manometer, and an oil vacuum pump. The material to be dried is packed under sterile conditions in ampules and frozen by dipping into acetone and dry ice at a temperature of  $-72^{\circ}$  or into liquid air at a temperature of  $-180^{\circ}$ . The drying process takes place under high vacuum. The ampules with the dried cultures are vacuum-sealed with fire.

In some medical bacteriological institutes manifold vacuum apparatus are at present the principal ones used for preparing dry living vaccines, diagnostic sera and bacterial cultures. In one

of the largest bacteriological institutions of the Soviet Union, the Central Institute of Epidemiology and Microbiology, they use manifold vacuum apparatus (Figure 8) constructed in the institute's shops and in the medical apparatus factory from K. Ye. Dolinov's drawings.

The material to be dried in these apparatus is packed into sterile ampules and prefrozen in a cooling mixture consisting of ice and table salt at a temperature of  $-20^{\circ}$ . The ampules with the frozen preparation are attached to the manifold of the apparatus. Drying takes place under a high vacuum (50-100 microns). The degree of vacuum in the system is determined by a Dolinov mercury vacuum manometer. Water vapor from the material being dried is absorbed by calcium sulfate in the chamber to which the manifold is attached.

In the section on neurotropic infections of the Institute of Neurology the method of drying in a manifold vacuum apparatus is likewise used for preserving a number of the filtrable viruses. Water vapors are absorbed by calcium sulfate. The dry viruses are kept in vacuum sealed ampules.

Using our designs, N. P. Sheptun, a mechanic, constructed a manifold vacuum apparatus (Figure 9) at the State Veterinary Preparations Research Institute. With this apparatus we have dried bacterial cultures, filtrable viruses, and diagnostic sera.

The apparatus consists of the following basic parts: a metal chamber for the calcium sulfate, a metal manifold with outlets, a shallow tray for the cooling mixture, a vacuum manometer, an oil vacuum pump, and a series of metal connectors to which the individual vials are attached.

The manifold vacuum apparatus is a hermetically sealed system when in operation. As a result of the high vacuum (50-100-200 microns), created in the whole system by the vacuum pump, the frozen state of the material being dried is maintained as long as there is any moisture. A mixture of snow or ice with table salt is used in freezing biological preparations and bacterial cultures. The degree of pressure in the system is determined by the Dolinov vacuum manometer.

For a moisture absorbent the apparatus employs calcium sulfate which is an excellent chemical absorbent in the desiccation of bacterial cultures, filtrable viruses, and diagnostic sera. The use of calcium sulfate as an absorbent in this case is profitable since the microorganisms, filtrable viruses, and diagnostic sera are dried in small batches and quantities.

#### Chamber-Type Vacuum Apparatus

More effective for drying biological preparations in large quantities is a chamber-type vacuum apparatus since it has a large capacity. However success in using the chamber apparatus depends on the successful selection of a method for condensing the water vapor.

As already pointed out, the use of chemical absorbents for these purposes is not efficient. For this reason the following methods of moisture removal are employed in the desiccation of biological preparations. The first method is the condensation of water vapor at low temperature in the form of ice on the outer surface of a coiled tube cooled by an effective cooling agent (ammonia or Freon). The second method is the removal of water vapor into the atmosphere without the use of chemical absorbents

or low temperature. The second method is the most practical and inexpensive. However, in order to use this method you need to have a high vacuum pump with an adapter for constantly removing from the oil the water which collects during the drying process.

Attempts of some foreign investigators to make a vacuum chamber apparatus with a refrigerated condenser have been unsuccessful in view of the small capacity of the apparatus designed and their imperfections.

Titov and Kalashnikov in 1939 proposed a chamber-type vacuum apparatus for the desiccation of biological preparations from a frozen state (Figure 10). During desiccation the water vapors are condensed in the form of ice on the inner wall of a metal pipe located between the chamber of the apparatus and the vacuum pump. The condenser pipe is cooled by a mixture of ice and table salt at  $-20^{\circ}$ . The drying process for large batches of serum and plasma lasts for 5 days. This apparatus has not become widely used as the result of serious shortcomings. This is especially applicable to the principle of condenser design and the cooling method.

We know from practice that for the most intensive condensation of moisture in the form of ice the condenser must be cooled to a minimum of from  $-28^{\circ}$  to  $-35^{\circ}$ . In Titov and Kalashnikov's apparatus however the condenser is chilled at  $-20^{\circ}$ . In view of this the desiccation process lasts for 5 days.

In 1945 N. N. Titov, K. F. Zemlyannikov, and S. I. Didenko proposed an improved chamber apparatus for drying biological preparations from a frozen state (Figure 11).

The apparatus is a metal chamber of the large desiccator type. In the lower part of the chamber is placed the prefrozen preparation which during drying is cooled by a liquid cooling agent circulating from the ammonia compressor through a system of pipes. As needed, the chamber of the apparatus may be heated during drying to  $80^{\circ}$  by illuminating gas or an electric unit. In the upper part of the chamber is located a metal coiled tube for condensation of water vapors to ice. This coil is cooled by the circulation of liquid ammonia at  $-27^{\circ}$  to  $-30^{\circ}$  from a compressor. The degree of vacuum in the apparatus can be determined by a Bolinov vacuum manometer. It takes 3 days to dry large batches of biological preparation frozen on the inner peripheral surface of a flask in the form of a shell. The amount of material which can be dried simultaneously cannot exceed 3 lit.

The advantage of this apparatus consists in the fact that the condenser coil is cooled at a lower temperature, while the drying chamber is heated. As a consequence the drying process is accelerated. However as a defect we must point out that the coiled tube for condensing the water vapor is not mounted separately but in the chamber where we have the material for drying. This situation, it seems to us, cannot fail to influence the length of drying time and increase the amount of residual moisture in the preparation because of the extreme nearness of the coiled tube to the material to be dried. If the condenser coil were located in a separate chamber alongside the full effectiveness of the drying process would be attained much more easily.

In the literature we have a description of a vacuum apparatus with a glass chamber which permits the drying of a large amount of human blood plasma (Figure 12). The design of the apparatus was later modified and the number of chambers increased to 8. The apparatus is a vertical circular steel chamber measuring 91x182 cm.

In the lower part of the chamber is a coiled pipe through which circulates a solution cooled to  $-44^{\circ}$ . In the upper part of the chamber is a receptacle with 180 holders in which the bottles with the prefrozen plasma are placed. The holders are wound in a spiral for electric heating during desiccation. The degree of vacuum in the apparatus is determined by a vacuum manometer. The drying process lasts 7 days, that is, 3 days in the first chamber with the coil and 4 days in the second chamber over phosphoric anhydride. The supplementary drying over a chemical absorbent is probably induced by the fact that the residual moisture in the plasma is markedly greater than indicated by the authors. An increase in the amount of moisture in the plasma may take place when the chamber of the apparatus is opened due to the presence of the coil on whose surface the water vapor has condensed in the form of ice. This situation as well as the very long drying time (7 days) makes the apparatus of slight value. The plasma meant for desiccation is frozen at a temperature of  $-17^{\circ}$  in a special apparatus. For this purpose a specific amount of serum is poured into upright bottles while sterile conditions are maintained. As the bottles are rotated at 890 rpm their surfaces are cooled by a stream of cold air. The plasma freezes on the inner surface of the bottles, spreading out in a thin layer.

An apparatus of another design consists of 2 metal chambers connected by a pipe through which the water vapor is removed from the drying material to the condenser (Figure 13).

The plasma is frozen in advance on the inner surface of a flask at a temperature of  $-30^{\circ}$  to  $-35^{\circ}$ . A special pumping apparatus is used for freezing (Figure 14). The flask is placed in alcohol cooled by Freon 12 gas.

After freezing, the flask with the plasma is placed in the drying chamber in special holders made of copper. The water vapors condense as ice on the surface of a coiled tube at a temperature of  $-30^{\circ}$  to  $-35^{\circ}$  in the cooling chamber. The coil of the condenser is cooled by Freon 12 gas from a compressor.

In order to hasten the drying process in the drying chamber heat is supplied by hot water circulating in a jacket around the chamber at a temperature of approximately  $80^{\circ}$ . The drying process in the apparatus is recorded automatically. The degree of vacuum is determined by a mercury vacuum manometer. The plasma drying process lasts for 24 hours. The capacity of the apparatus varies. There is a laboratory one for 1.8 lit, a pilot type for 72 lit, and a commercial type for 21.6 lit. As a defect of these apparatus it must be pointed out that the pipe connecting the chambers for removing water vapor is too long, is not large enough and has sharp bends in it. This slows down the drying process considerably.

In 1940 a vacuum chamber apparatus was proposed in which the water vapor from the material to be dried was drawn off by a high vacuum pump (Figure 15). The vapor passes directly into the oil circulating in the pump. The oil passes continuously through a centrifugal purifier, which is a centrifuge for removing the water. After the water is extracted, the oil passes directly back into the vacuum pump, etc.

The use of these pumps permits us to dry material with a comparatively low vacuum (4,500 microns of residual pressure), at the same time guaranteeing that the material will remain in a frozen state. In order to speed up the drying process warm water circulates through the jacket of the drying chamber.

In 1945 another type of vacuum chamber apparatus was proposed for drying human blood plasma, penicillin and other biological substances (Figure 16). The capacity of the apparatus enables us to prepare dry biological preparations in large quantities.

Before drying, the material is frozen directly in the drying chamber. For this purpose a cooling agent is circulated in the jacket and hollow tubes of the chamber. After freezing is finished, the vacuum pumps are turned on and the drying process begins. If it is necessary to hasten the drying process, as for example, when plasma or serum is undergoing desiccation, the cooling agent is removed from the jacket and hollow tubes of the drying chamber and replaced by hot water at a temperature of 60-75°C.

The water vapors are condensed in a condensation chamber on the surface of coiled pipes at a temperature of -27° and lower. The coils are cooled by Freon 12 gas or Freon 22 from compressors. The high vacuum also contributes to the creation of a low temperature in the condenser.

At the Research Institute of Epidemiology and Hygiene of the Soviet Army living bacterial vaccines are dried in a vacuum apparatus designed by Karneyev, Del'nik, Grudnikov, and Chernykh. The vaccines are freeze-dried using a high vacuum pump (models 412 and 212). This pump removes vapor from the material to be dried directly into the atmosphere without the use of any chemical absorbents or mechanically cooled condenser. At the institute they are likewise using a manifold apparatus for drying, using calcium sulfate as an absorbent.

In 1944 a report was published on the use of infrared rays in a special apparatus for drying biological products from a frozen state (Figure 17). The action of infrared rays on frozen serum markedly accelerates the drying process, with the latter proceeding evenly since the rays act throughout the whole layer of the preparation.

In modern drying apparatus the walls of the chamber are heated by electric elements, steam, hot water, etc, in order to speed up the drying process. However these heating methods demand especially equipped apparatus and do not bring about the most rapid desiccation process since in these cases the heat acts only on the surface layer of the frozen preparation. The deeper layers of the preparation become heated as the moisture is removed from the upper layers. For this reason infrared rays are the best type of heat energy that can be used for drying sera from a frozen state. Apparatus with infrared radiation are as yet not widely used in practice.

At the Institute of Epidemiology and Microbiology imeni N. F. Gamaley and the Central State Control Institute imeni Tarasevich small chamber apparatus are used alongside the manifold apparatus. The material to be dried is placed on the bottom of the chamber. The moisture is absorbed by calcium sulfate which is placed in a special can in the same chamber over the material being dried.

At the State Institute for Control of Veterinary Biological Preparations mechanical engineer N. P. Sheptun made a chamber-manifold apparatus (Figure 18).

This apparatus is a hermetically closed metal chamber with a large metal manifold attached to it. We introduced certain changes into the design of the chamber-manifold apparatus so that we would be able to use both the chamber and the manifold method for drying preparations in it. In the first case, the manifold is cut off from the chamber by inserting inside the flange rubber and metal plates which are tightened down by the counterscrew of the manifold. When the manifold method is being used for drying both plates are removed from the flange and thus the manifold communicates with the chamber containing the calcium sulfate.

The preparation, use, and regeneration of calcium sulfate (gypsum) for the drying process in a chamber-manifold apparatus are the same as those used for drying in a manifold-type apparatus.

### CHAPTER III. DRYING METHODS

The methods to be used for preserving bacterial cultures, filtrable viruses, and biological preparations for a long time by drying must meet 2 basic requirements. First, the drying process must have no harmful effect on the biological preparation. Second, the dry preparations must retain their biological properties for a long time.

Earlier methods for drying in vacuum desiccators without preliminary freezing and in drying cabinets using artificial heat led to a rapid loss of the active properties of the biological substances. The decrease in activity, according to the findings of certain investigators (S. S. Rechmenskiy, A. Klimentova, E. Kruk, and G. Yarmol'chuk), takes place as the result of denaturation of protein. In view of this fact the serum, for example, does not have total solubility. The loss of total solubility in dry serum causes not

only changes in its physical and chemical properties but diminishes its activity. Other investigators explain this by the fact that when drying takes place in a desiccator without prefreezing there is a concentration of the salts and solid layers are formed which decrease in weight in the course of several months. In their opinion, this decreases the activity of the preparation.

It is doubtful that this is true. The loss of active properties in biological preparations, dried without freezing, apparently takes place because the protein material being dried has a great percentage of residual moisture bringing on an oxidation process while the factors of protein denaturation determine the incomplete solubility of the preparation, diminishing its quality.

Vacuum apparatus are being widely used in practice which makes possible the drying not only of small quantities of bacterial cultures, viruses and diagnostic sera (manifold apparatus) but also large quantities of serum and vaccine preparations (chamber apparatus). In both the manifold and chamber-type apparatus drying takes place from a frozen state under high vacuum. It must however be pointed out that the principal and decisive factors in this process are the factors of condensation or absorption of moisture from the material being dried and the freezing factor.

In making dry preparations in a manifold apparatus or in a small chamber apparatus, chemical agents, such as calcium sulfate, phosphoric anhydride, calcium chloride, and certain others, are used as moisture absorbents.

In large chamber apparatus a special condenser with a compressor for cooling is needed for moisture removal.

In both cases absorption or condensation and prefreezing of material are of equal importance.

#### Importance of the Freezing Factor in Desiccation

As has already been pointed out, drying biological substances from a liquid state leads to a marked loss of active properties as a consequence of incomplete moisture removal and denaturation of protein. The situation is quite different when biological substances and living organisms are dried from a frozen state. In this case the active properties of the dry biological preparations are retained completely and the number of viable bacterial cells diminishes only slightly.

The dry porous mass of dry serum preparations testifies to the fact that the protein has become condensed. This is likewise confirmed by the small decrease in its original volume. Proteins subjected to momentary freezing evidently do not regroup but remain in the state in which they were before freezing. This also eliminates denaturation of the proteins which contain the active principle and irreversible changes are almost entirely absent. Eradication of denaturation of proteins and concentration of salts during drying are also helped by the fact that moisture from a frozen preparation is removed without passing through the liquid phase. Thanks to this very fact, the proteins of serum preparations do not become thicker and remain in a rarified, crystalline state as we can easily observe through microscopy. In addition, during freeze-drying the process of moisture removal proceeds much faster, thanks to the action of the low temperature, the presence of a vacuum, and the equality of pressure over the evaporation surface of the frozen preparation and the moisture absorbent.

Freeze-drying makes it possible to produce dry biological preparations or bacterial cultures and viruses with exceedingly small amounts of residual moisture. This is achieved by the fact that with proteins in a rarified condition their surface area is greatly increased which in itself facilitates more thorough desiccation. This drying method for biological preparations assures rapid and total solution in water and preserves their original physical properties.

Freezing liquid substances before drying is likewise necessary in order to prevent foam formation when a deep vacuum is set up in the apparatus. The foam formed in the ampules greatly impedes the drying process and sometimes contributes to passage of material into the absorption agent. A gradual increase in vacuum cannot prevent this undesirable phenomenon since it is practically impossible to determine the degree of foaming in each ampule when drying takes place in a manifold apparatus with such a large number of connections and ampules.

For some time it has been believed that serum preparations and bacterial cultures and viruses undergo more thorough drying if they are frozen at a temperature of  $-75^{\circ}$ . However the majority of Soviet investigators (A. Klimentova, R. Kruk and G. Yarmol'chuk, K. Ye. Dolinov, S. G. Kolesov, B. S. Del'nik, N. N. Titov, etc) have established that fact that carbon dioxide and liquid air can be successfully replaced by a less expensive and more readily available cooling mixture consisting of snow or ice and table salt which will produce a temperature of  $-20^{\circ}$ .

Among the chemical substances which produce low temperatures, mention must be made of potassium chloride, calcium chloride, ammonium chloride, ammonium nitrate, sodium nitrate, sodium chloride, ammonium sulfate, and crystalline calcium chloride.

If these salts are added in proper proportions to snow, the following temperatures in the cooling mixture may be produced.

Name of Salt	Parts by Weight per 100 parts of snow	Temperature of mixture (degrees)	Name of Salt	Parts by Weight per 100 parts of snow	Temperature of mixture (degrees)
KCl	30	-11	NaCl	33	-21.2
CaCl <sub>2</sub>	30	-11	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	62	-19
NH <sub>4</sub> Cl	25	-15.8	CaCl <sub>2</sub> ·6H <sub>2</sub> O	82	-21.5
NH <sub>4</sub> NO <sub>3</sub>	60	-17.3	CaCl <sub>2</sub> ·6H <sub>2</sub> O	125	-40.3
NaN <sub>3</sub>	59	-18.5	CaCl <sub>2</sub> ·6H <sub>2</sub> O	143	-55

From the data supplied in the table we can see that table salt is the most suitable for making a cooling mixture since its addition to snow in a comparatively small amount lowers the temperature of the mixture to -20° or -21°. In desiccation in manifold apparatus such a temperature is satisfactory.

When drying is taking place in small chamber apparatus it is best for cooling the outside of the chamber to use solid carbon dioxide and alcohol which produce a temperature of -75°. Such a low temperature is necessary since the material being dried is separated from the cooling mixture by the walls of the chamber.

Freezing agents, such as snow and table salt or solid carbon dioxide and alcohol, are quite suitable both before drying and during desiccation in a manifold or small chamber apparatus. These same freezing agents also may be used for prefreezing for a large chamber apparatus. However another cooling agent and another cooling system are needed in order to maintain the material in a frozen state during the first stage of drying. For instance, desiccation in a large chamber apparatus is carried out by using a compressor and such cooling agents as ammonia, Freon (12 and 22), and others. The cooling agent circulates in special hollow jackets and hoses of

the apparatus. The effectiveness of this cooling method is very great since the cooling agent drops the temperature of the preparation from  $-24^{\circ}$  to  $-32^{\circ}$  and lower.

#### Calcium Sulfate as a Moisture Absorbent

The intensity of the process for drying liquid protein substances from a frozen state depends to a large extent on the quality of the chemical absorbent when desiccation takes place in manifold and small chamber apparatus and on the effectiveness of the refrigerated condenser when drying takes place in large chamber apparatus.

When drying small quantities of preparations or bacterial cultures in desiccators we usually use phosphoric anhydride, calcium chloride, and certain other chemical agents. Recently calcium sulfate has been widely used in vacuum apparatus. The moisture-absorbing capacity of all these chemical substances varies as we see from the following data.

Drying Substance	Amount of Water Vapor Remaining in 1 lit of air at $25^{\circ}$ (in mg) [Note]
Phosphoric anhydride	0.000025
Potassium hydrate (liquated)	0.002
Calcium sulfate (gypsum)	0.004
Calcium chloride (granulated)	0.14
Sulfuric acid (95.1%)	0.3

[Note] The drying capacity of a chemical substance is determined by observing the following conditions. Air, saturated in advance with water vapor, is passed over the drying substance at a rate of 1-3 lit per hour at  $25^{\circ}$ )

As we see from the table, phosphoric anhydride has the greatest capacity for absorbing moisture, followed by potassium hydrate and gypsum. However, the first 2 absorbents, despite their high absorptive capacity, have surface layers that quickly become saturated with moisture, thus rendering them inactive.

Phosphoric anhydride, for example, is used by many investigators as an auxiliary absorbent in drying serum preparations in chamber apparatus with a refrigerated condenser. The use of phosphoric anhydride for this purpose is quite practical since the principal portion of the moisture (96-98%) is removed from the preparation by primary drying. For this reason the great absorption capacity of the phosphoric anhydride per unit of weight assures supplementary desiccation of the preparation with a reduction of residual moisture to 0.5-0.7%.

Calcium chloride is likewise active only until its surface layers are saturated with moisture and it is used in those cases where a small amount of material is being dried. The same may be said of sulfuric acid.

Calcium sulfate (gypsum) with a pronounced absorption capacity is not subject to the same solution of its surface layers. This property of gypsum provides for the entry of even the inner particles into the drying process. For this reason gypsum is considered a more valuable chemical absorbent for drying bacterial cultures, viruses, and certain preparations.

Calcium sulfate, an anhydrous substance, has a powder form. However, in its powder form it is of no use as an absorbent since only its surface layers take part in the absorption process.

In order to obtain the most surface with the purpose of increasing the moisture absorption capacity, calcium sulfate is used in the form of granules. Powdered gypsum is mixed with water until a paste is formed, after which it is allowed to set and when dried it is cut into small granules. Gypsum thus prepared is freed of its finest particles and dried in a drying cabinet at 180-200° until it reaches a constant weight. The primary gypsum drying process lasts 12-16 hours when the layer of gypsum granules spread out to dry is equal to 2.5-3 cm.

The regeneration of calcium sulfate following each drying cycle lasts 8-10 hours at a temperature of 180-200°.

The value of gypsum as a drying agent consists in the fact that the deep vacuum and great absorption capacity of the gypsum greatly increase the degree of passage of moisture from the material to the absorbent which makes for high grade desiccation. In addition, gypsum is easily regenerated (restored) after each drying cycle in the apparatus and remains useful for no less than 20-25 drying cycles. We must here take into consideration the amount of moisture absorbed by the gypsum during each drying cycle.

For example, our experiments established that when 10 ml of liquid are dried in the presence of 1 kg of gypsum the latter may be used for 20-25 cycles with regeneration following each drying cycle. If we use 20-30 ml of liquid per kg of gypsum it soon becomes inactive although it can be used to dry small batches of material (for example, bacterial cultures when the amount of moisture does not exceed 3 ml per kg of gypsum). However gypsum is an inefficient absorbent when large amounts of preparation are being dried.

As already indicated, each kg of gypsum is able to absorb 10, or a maximum of 20 ml of liquid. In view of this fact, gypsum becomes unsuitable for drying large amounts of biological preparations.

#### Desiccation Based on a Refrigerated Condenser

Recently, powerful chamber vacuum apparatus have come into use for drying large amounts of biological preparations. During desiccation in these apparatus the moisture condenses on the surface of the condenser coils, cooled by a compressor and Freon or ammonia bases. This condensation system to a great degree makes the drying of biological preparations less expensive and simpler since there is much less expense per unit of finished product.

In addition, in large-type chamber apparatus the preparation may be dried not only in small but in large vessels (100-200-300 ml). Desiccation in chamber apparatus necessitates considerable equipment. The drying chambers, for example, must be furnished with a special cooling and heating system. Shelves with cells or holders for the preparation are made of metal with a high degree of thermal conductivity.

The chambers are provided with automatic devices for determining and regulating temperature and vacuum.

The operation cycle of the condensation chamber is likewise regulated automatically by using a vacuum device and a thermocouple which maintain a specific temperature. In addition, the chamber is furnished with thermal elements capable of removing ice from the surface of the coils after the drying process is finished.

The low temperature in the condenser coil is maintained by the chamber vacuum which, so to speak, insulates the coil from the effect of the temperature of the outside air. For drying in large

chamber apparatus we use powerful vacuum pumps capable of pumping large volumes of air and of creating a deep vacuum in the apparatus. Without this, the drying process would drag out over a long time and some preparations, such as plasma and penicillin, can hardly be dried without a sufficiently low temperature.

The effectiveness of drying is likewise greatly influenced by the freezing factor. The lower the temperature during the initial drying phase, the more rapid is the extraction of moisture from the frozen material. Certain investigators believe that the drying of plasma and serum demands various freezing temperatures. For instance, for drying serum a temperature of  $-9^{\circ}$  to  $-12^{\circ}$  is sufficient at the start of the process, while only  $-20^{\circ}$  to  $-25^{\circ}$  will do for drying plasma.

#### Desiccation in Manifold Apparatus

The manifold vacuum apparatus (Figure 9) consists of a metal chamber for the gypsum, a metal manifold, and a series of connections. The apparatus is furnished with a Dolinov vacuum manometer and an oil vacuum pump. In operation the apparatus is a closed system, which makes it possible to produce a deep vacuum (50-100-200 microns).

The water vapors in a manifold apparatus are absorbed by calcium sulfate. The intensity of absorption is determined more by the lower moisture pressure at the absorbent than the pressure of saturated vapor at the evaporation surface of the material being dried. It must also be pointed out that the degree of the intensity of the motion of water vapor molecules likewise depends on the distance of the material from the absorbent. It has been proven for practical purposes that the more bends in the line leading the

moisture through the apparatus, the slower the drying process. However, when the apparatus is a fully hermetically closed system the drying process proceeds rather rapidly, especially if the material is dried in small batches.

The speed of the drying process is related to the intensity of moisture evaporation, that is, to the removal of water vapor from the frozen material. For this reason in certain cases the rate of vapor removal can be increased by allowing heat to reach the frozen material in the ampules. In order not to cause liquefaction of the frozen material the heat must act only on the outer surface of the ampules. However heat cannot be employed in all cases. For instance, heat may be permissible in drying serum preparations while heating might weaken the virulence of bacterial cultures and filtrable viruses.

The use of a manifold vacuum apparatus at the State Control Institute of Veterinary Products of the Ministry of Agriculture USSR and at a number of institutes belonging to the Ministry of Public Health has shown that this apparatus may be used for successfully drying bacterial cultures, viruses, and diagnostic sera. The dry biological preparations dried in these apparatus answer all requirements of existing regulations. Drying in a manifold apparatus completely eliminates all possibility of the entry of foreign bacteria into the dried material. This is especially important in the preservation of standard strains. In addition, it is easy and convenient to vacuum-seal the ampules in this apparatus without recourse to additional manipulations of joining the ampules to the connectors and to the manifold, such as are necessary in drying with a chamber apparatus.

The drying process in a manifold apparatus consists of the following. After the bacterial cultures, viruses, or diagnostic sera have been packed in ampules and the latter fastened to the connecting tubes by means of vacuum-resistant rubber, the preparations are frozen at a temperature of  $-20^{\circ}$  in a cooling mixture consisting of snow and table salt. After 10-15 minutes the connecting tubes with the ampules are attached to the manifold apparatus. The drying takes place under a vacuum of 50-100-200 microns pressure with the ampules with the frozen material sitting in a shallow tray containing cooling mixture at  $-15^{\circ}$  to  $-17^{\circ}$  for preserving the material in a frozen state during the first 3-4 hours of drying. If the vacuum pump is capable of pumping off air from the system satisfactorily, drying may take place at an even lower vacuum (500-700 microns).

A high vacuum maintains the material in a frozen state and accelerates the drying process. For this reason it is necessary to see to it that the system is hermetically sealed since sometimes a very small opening is enough to diminish the vacuum in the entire system.

After the apparatus has been in operation for 3-6 hours the first phase of the drying process is considered finished. The shallow trays with the cooling mixture are then removed and the drying continues at room temperature.

Desiccation of bacterial cultures usually lasts for 10-12 hours while that of viruses and diagnostic sera lasts 24 hours. It must be pointed out that these periods of time, which we use in our experiments, are the maximum and are necessary for the most thorough drying. We know from the work practices of other investigators that bacterial cultures may be dried in small batches in even less time (5-6 hours) but this length of time does not always give assurance of thorough drying.

Bacterial cultures, viruses and sera dried by the system outlined usually contain a minimal amount of residual moisture (from 0.4-0.7 to 1.0-1.5%). Residual moisture is determined by supplementary drying in a cabinet at 105° until a constant weight is reached or in a desiccator under vacuum over calcium chloride which has itself been dried to a constant weight. It must however be pointed out that these methods for determining residual moisture do not all give the same indexes. For example, when determined in a drying cabinet at 105° the percentage of residual moisture is usually higher, since at this temperature a portion of the protein may be consumed. Testing dried cultures over calcium chloride usually gives a smaller percentage of residual moisture (0.4-0.7) although the process of complete drying in a desiccator under vacuum lasts for 3 days. The more accurate determination of residual moisture is achieved by supplementary desiccation in a desiccator over phosphoric anhydride under vacuum.

There is an opinion current that any determination of the degree of desiccation gives arbitrary results since in all cases the dry substance may probably contain a certain amount of moisture in a bound form. However a dry substance does not undergo a marked loss in weight when phosphoric anhydride acts upon it.

After drying is finished the ampules with the material are vacuum-sealed over a flame. It is best to seal the ampules with a 2-flame gas-oxygen burner. This type of sealing is more thorough. The spot where sealing takes place is covered with nitrolacquer. The ampules are tested for vacuum with a Tesla apparatus and stored in a refrigerator.

In our experiments the dried bacterial cultures, viruses, and diagnostic sera were usually stored at room temperature (17°, 20°, 27°). These conditions are not ideal since some instable cultures must be kept in a dry form at a temperature of 2-5°.

however 6 years of personal observations and experiments of certain laboratories of our institute as well as other investigators indicate that at room temperatures of 17-20° it is possible to keep not only dry serum preparations but bacterial cultures and viruses.

#### Desiccation in a Chamber-Manifold Apparatus

When the chamber drying method is used the material is placed in a special container on the bottom of the chamber. In the upper part of the chamber another container holds gypsum to act as a moisture absorbent. The material to be dried is packed into ampules which are closed with a sterile cotton wad and frozen at -20° in a cooling mixture.

In order to keep the material from thawing, especially at the beginning of the drying process, the chamber is cooled by a mixture made from snow and table salt. However, it is better to use a cooling mixture of solid carbon dioxide and alcohol which produces a temperature of -75°. This temperature is more effective for cooling the chamber.

The drying process in the chamber lasts from 6 to 24 hours depending on the amount of material poured into the ampules. The absorption norms for gypsum remain the same as for drying in a manifold apparatus. After the drying process is finished the ampules with the material are connected with a vacuum washer to the connecting tubes and the manifold in order to produce a vacuum in them. The ampules are sealed over flame when the vacuum reaches 100-200 microns.

The possibility of producing a high vacuum in the chamber and the total hermetic condition of the apparatus are the prerequisites for successful desiccation. The use of cotton stoppers in the ampules for the prevention of contamination by foreign bacteria lengthens the drying process to a certain extent. However when drying small amounts of material which are packed in small units the drying process takes place rapidly. The manifold method may be used with the same apparatus. In this case the chamber is completely filled with gypsum. The stopper which closed off the pipe leading to the manifold is removed and the ampules with the prefrozen material are attached directly to the connecting tubes. The drying process takes place as in the usual manifold apparatus.

#### Desiccation in a Chamber Apparatus

As distinct from the manifold apparatus, the chamber apparatus may be used for drying large amounts of material. For this reason the use of powerful chamber apparatus in biological preparation plants permits the production of the quantities of dry preparations needed for extensive use in practice.

The chamber vacuum apparatus consists of one or 2 metal chambers of the cabinet type and one circular refrigerated chamber. The biological material is again dried from a frozen state under high vacuum. Separate tubes for removing water vapors from the material being dried makes it possible to load the drying chambers as the material arrives. In addition, each of the drying chambers may be included in or cut off from the drying process by special closures (stoppers), located in the pipes connecting the drying chamber with the condenser. The entire drying process in the apparatus is regulated automatically.

The material is frozen before drying in a special apparatus (Figure 14). It may also be frozen in the drying chamber before desiccation starts if this chamber is furnished with a system of hollow jackets and hoses through which a cooling agent may circulate from a compressor.

After prefreezing, by rapid rotation of the flasks in a vertical position in a circulating freezing apparatus or in a room cooled to  $-17^{\circ}$ , the material is placed in the drying chamber where it undergoes desiccation. When frozen directly in the drying chamber the preparation is cooled by ammonia or Freon which enters the chamber from the compressor. After freezing (this takes only several minutes), a deep vacuum is set up in the whole apparatus by powerful high vacuum pumps.

In the chamber apparatus the drying process varies in length of time. For instance, if the material is dried in a small amount it usually takes 24 hours but if the material is packed in a large amount the drying process is lengthened to 36 hours or even to 2-3 days. In addition, a great role is played by the size of the surface of the frozen preparation. For instance, if we pour 200 ml of serum into a 300-ml flask and freeze the preparation in the form of a lining on the inner walls of the flask the drying process in a powerful chamber apparatus may be finished in 24 hours since the greater the surface of the frozen material the less time it takes for desiccation.

While it is possible to successfully vacuum-seal cultures and viruses in ampules, it is difficult to create sufficient vacuum where large batches of preparation are frozen in flasks. In this case some investigators recommend filling the flasks with purified

dry nitrogen which is a neutral gas. The flasks are filled with the gas directly in the chamber of the apparatus. This is done as follows. After the drying process is finished the vacuum pump is turned off and pure dry nitrogen is led into the chamber.

Thanks to the deep vacuum in the entire system and in the flasks, the nitrogen rapidly fills the latter. After this the flasks with the preparation are stoppered, daubed shut, and sealed.

The dry preparation is stored at a temperature of 5-15°.

Before being used the dry biological preparation is dissolved in a sterile physiological saline solution or distilled water, adding the amount of liquid which the flask contained before desiccation started. However, in certain cases the preparations may be dissolved in different proportions. Immune sera, for example, are dissolved in a quantity of water equal to half or even 1/3 the original volume of the preparation. Bacterial vaccines which are dried in the form of thick emulsions may be dissolved in a larger quantity of water than there was before desiccation.

#### CHAPTER IV. ANABIOSIS IN MICROORGANISMS

"Wherever there is life, we find that it is associated with some protein body, and wherever there is a protein body not in the process of decomposition, we are, without exception, seeing manifestations of life." -- F. Engels

Problems relating to the state of bacterial cultures, viruses, and biological preparations when preserved in a dry form have been treated very little in the literature. Information on hand from a number of investigators in this field deal only with factors of the viability of bacteria and viruses and their biological properties. However the authors do not in their reports treat the problems which

clarify the state of bacterial cells in a dry form. For this reason, in studying the properties of dry bacterial cultures, we believed it necessary to analyze and enlighten the theoretical problems which deal with the state of bacteria in a dry form as well as when they are acted upon by low temperatures.

As a theoretical prerequisite to the possible duration of the preservation of the viable properties of labile microorganisms we used in our experiments the factor of anabiosis of sporogenous bacteria and certain protozoa which become encysted during freezing or desiccation under natural conditions.

Anabiosis in the proper sense means a return to life, a resuscitation. However the word anabiosis usually suggests not revivification after preceding death but latent life or a temporary cessation of life processes.

We find it necessary to point out that from our point of view anabiosis of microorganisms in experimental desiccation and preservation under vacuum must obviously be thought of as a temporary cessation of life. And this is the point of view from which problems of anabiosis and our critique of views of other authors on this subject will be treated.

The materialist approach in the field of biology indicates that the role of the environment is decisive in the life of organisms. For this reason, in studying the conditions under which bacterial cultures are dried and preserved we are guided by the doctrines of I. V. Michurin and T. D. Lysenko about the effect of the external environment on the living organism. For instance, whereas moisture, nutritive substances, and atmospheric oxygen are necessary if aerobic bacteria are to be preserved under ordinary conditions, an entirely different medium is required if their biological properties are to be preserved for a long time in a dry form.

We know that the evolutionary development of organisms includes principally the factors representing the effect of the surrounding medium, that is, the conditions under which they continue to exist. Under the influence of the environment the organisms gradually change their biological properties by developing in themselves those features which are the most resistant to the external conditions. Such an evolutionary development, for example, is a feature of the rotifer *Philodina* and the tardigrade *Macrobiotus* which, by living under natural conditions among mosses and lichens, acquired during their evolution the capacity of preserving their viability for a long time during freezing or desiccation. Like capacities to resist unfavorable environmental conditions have been acquired by many other plants and animals (algae cultures, amoebae, infusoria, certain nematodes, etc).

By analyzing historical data on anabiosis in plant and animal organisms following freezing and desiccation one may assume that even labile nonsporogenous bacteria have the capacity to withstand such factors under experimental conditions where certain additional substances are being created. This theoretical hypothesis could be expressed where there was a materialist concept of manifestations of anabiosis, that the life of bacteria in a dry or frozen state under experimental conditions takes on completely different forms, that is, is extremely retarded, inhibited, or even temporarily comes to a halt, as apparently takes place in cases where bacteria retain their viability in soils of the permanent frost zone.

Scientific literature contains a number of reports on the very great resistance of bacterial cultures and viruses to

experimental desiccation and freezing. However the explanations for the causes of this resistance and the state of these creatures in the dry and in the frozen state are very few. For this reason the problem which this book poses regarding anabiosis in bacteria, when the latter are preserved in a dry or frozen form, as a manifestation of a particular form of existence of minute organisms must be a subject not only for discussion but for further study.

In considering the problems of the great resistance of microorganisms to freezing and desiccation we must give some attention to G. M. Bosh'yan's book O prirode virusov i mikrobov [On the Nature of Viruses and Microbes] which was recently published, 1950, Moscow, Medgiz.

During a study of equine infectious anemia, as G. M. Bosh'yan reported, he established the fact that the filtrable virus of this disease is connected to the proteins of the organism, the nucleoproteins.

When the connection between the virus and the proteins is disrupted, the virus may be transformed either in the organism or in the test tube into a bacterial form. The author later established a similar feature in studying certain other filtrable viruses. This situation permitted G. M. Bosh'yan to come to the conclusion that filtrable viruses are a form of existence of microorganisms. If only the surrounding medium is changed, or the metabolism of the animal cells change following death, a change in the form of the pathogen develops and it is converted from an invisible (filtrable) form into a visible bacterial form.

By studying therapeutic and prophylactic sera, allergens and

antibiotics, G. M. Bosh'yan established the presence in them of filtrable forms of those microorganisms from which they were derived. As there was later confirmation of this tenet, the existent concept of the sterility of these preparations was changed radically. In addition this would point up the extreme stability of the filtrable forms of microbes. For instance, manifestations of the resistance of filtrable forms of microorganisms are especially phenomenal when we derive from them the original cultures of microbes from which such biological preparations were produced as tuberculin, mallein, brucellysate, and brucellohydrolysate. And these preparations were subjected to great effects by 4 cycles in an autoclave (mallein, tuberculin) and a session lasting many hours in the autoclave (brucellohydrolysate). In addition, as the author reported, he also demonstrated phenomena of great resistance of a number of microorganisms to chemical substances. For instance, bacterial and virus vaccines treated with formalin are not, in Bosh'yan's opinion, killed, as believed until recently, but only inactivated. If bacterial vaccines treated with formalin are given further proper treatment the original culture may be recovered, although it has but slight virulence.

The revivification of certain creatures subject to freezing and drying was observed more than 240 years ago. For instance, the first revival of rotifers when water was added to dry sand taken from an eavespout was observed by Antonj Leeuwenhoek (1701). Later such phenomena were observed in rotifers, nematodes, and tardigrades by other investigators (Nidhem, Spalanzani, and others). However these phenomena were not correctly interpreted. At the end of the nineteenth century there were once more many reports on this problem and Preyer (1873) identified the revival of rotifers,

tardigrades, and nematodes, heretofore known as "apparent death," "lethargy," or "numbness," as being anabiosis, that is, when an organism in a lifeless dry state is capable of revivification if there is a change in environmental conditions. Claude-Bernard held to the same opinion. However, despite the proposal of the new term "anabiosis," investigators of the time, including Preyer, could not furnish a scientific basis for the factor of the prolonged retention of viable properties in rotifers, tardigrades, and nematodes when they were kept in a dry state or under low temperature.

#### Anabiosis in Freezing

The study of anabiosis when living creatures are frozen was not systematically or scientifically studied until the beginning of the present century. An incentive was the research of the Russian scientist professor P. I. Bakhmet'yev in the field of the anabiosis of insects when frozen, published in 1899, 1900, and 1912.

Believing that the problem of the body temperature of insects and their condition at different temperatures, especially when frozen, had been given little attention, P. I. Bakhmet'yev took up the study. As a result he had soon developed a method for determining temperature in insects by using a thermoelectric thermometer. By using the thermoelectric thermometer which he had made, P. I. Bakhmet'yev quite accurately determined the body temperature of insects, at first under ordinary conditions and later at low temperatures.

By studying temperature conditions during freezing, P. I. Bakhmet'yev discovered a new phenomenon in the insect organism, the rapid temperature change with the preceding phase of overcooling.

By placing butterflies in a chamber chilled to  $-12^{\circ}$  to  $-22^{\circ}$ , the author established that their body temperature at first drops to zero and then after some time to  $-10^{\circ}$ , after which there is a sudden rise in temperature to  $-1.5^{\circ}$ . After this the body temperature of the insect again drops gradually and reaches the temperature of the cooled chamber.

By studying these phenomena, P. I. Bakhmet'yev determined that freezing the fluids of the insect organism does not take place at the time of the first drop in temperature to the critical point K, the so-called phase of super cooling, (super cooling of liquids is a purely physical phenomenon which is observed when a liquid is cooled evenly and when crystals of ice have not yet appeared in it) but during the second drop after the sudden temperature change, from point N to point T, that is, to a temperature of  $-9.4^{\circ}$  (Figure 19).

During the initial stage of cooling when the temperature reaches the critical point K, the insect merely becomes numb and if it is removed from the cooling chamber it returns to life. Later after the sudden temperature change there is a gradual freezing of the body's fluids, and the insect's body temperature drops to  $-10^{\circ}$ . If at any point along the curve between points N and T with the temperature at from  $-1.5^{\circ}$  to  $-9^{\circ}$  the insect is returned to ordinary conditions it returns to life and revives, although slowly. But if the insect is kept until the body temperature reaches  $-10^{\circ}$ , that is, to point T, the "death point," the insect dies.

Through special calorimetric calculations P. I. Bakhmet'yev determined that at a temperature of  $-4.5^{\circ}$  the fluids in the insect are completely frozen. For this reason the author believes that the state of the insect between points A and T, that is, during the

cooling phase between  $-4.5^{\circ}$  and  $-9^{\circ}$ , is anabiotic. Thus, as the result of these studies, the orderly theory of anabiosis in insects when frozen was created.

however it is necessary to point out that P. I. Bakhmet'yev based his theory on the fact that the insect may undergo total freezing during the phase from  $-4.5^{\circ}$  to  $-9^{\circ}$  when all the body's fluids are converted to a solid state and manifestations of life are completely terminated. But, as we see later, anabiosis in freezing takes place in insects only when the body is in a stage of super cooling and at the beginning of the appearance of ice crystals when there is still internal respiration, slow though it may be.

P. Yu. Shmidt introduced a significant correction in Bakhmet'yev's theory of anabiosis in insects when frozen. For instance, he did not confirm Bakhmet'yev's statements on total freezing of body fluids at  $-4.5^{\circ}$ . At this temperature not all the body fluids freeze and consequently anabiosis takes place under somewhat different temperature conditions.

The condition of anabiosis, according to P. Yu. Shmidt, in frozen insects may be noticed during super cooling, that is, from zero to point K, during the brief elevation in temperature, the so-called sudden change in temperature, from point K to point A, and during initial ice formation, between points A and  $T_1$  (Figure 20).

N. I. Kalabukhov, in his work, Spyachka zhivotnykh [Animal Hibernation] (1936), indicates that insects which revive with the coming of spring do not undergo total freezing during the winter. In a given case, as the author points out, the viability of the insects is retained only because a portion of the fluids in the

vessels and tissues in the body is not frozen. If the insect were subjected to total freezing with the hardening of all fluids, it would inevitably die. In a study of the action of low temperatures on the bee organism, N. I. Kalabukhov determined that during supercooling, that is, when the body fluids are not yet in a solid state, the metabolic processes do take place. By experimentation the author established the fact that the factor of nutrition contributes to increasing the resistance of bees to low temperatures. The author likewise demonstrated that with supplementary feeding of honey the resistance of the bees to cold increased markedly. For instance, the bees given extra feed and placed in a refrigerator at a temperature of  $-1^{\circ}$  survived to an extent of 50% after thawing and to the extent of 43% after  $-4^{\circ}$ . Only 24 and 9.5% respectively survived among the bees which had not been given supplementary feeding.

Of great interest is the work of N. L. Sakharov in which he reports on resistance to cold in insects. By studying the action of low temperatures on larvae of the winter moth, the author came to the conclusion, with the use of a dilatometer, that the formation of ice and the degree of the advance of the freezing point depends on the amount of water in the insect's body. For example, the author determined that in wintering larvae, containing 11% less water and 2.5% more fat than summer larvae, the formation of ice does not start until  $-11^{\circ}$ , and at  $-17.35^{\circ}$  only 15.22% of the water in the larvae's bodies changed to ice. When cold acts on larvae which have just left the nest and whose bodies contain more water and less fat, ice starts to form at  $-5.75^{\circ}$ , and at  $-7.8^{\circ}$  some 44.85% of the water has been converted into ice.

For instance, the experiments of N. I. Kalabukhov and N. I. Sakharov on the resistance of insects to cold indicate that the insect organism with a varying biological state is not all subject to the action of low temperature in the same degree. For this reason Bakhmet'yev's data on the obligatory and total freezing of insect fluids at  $-4.5^{\circ}$  and on the death point at  $-10^{\circ}$  are apparently not wholly correct.

Despite the statements of the majority of the investigators about the death of insects under total freezing there is likewise information to the effect that individual insects can withstand very low temperatures. For instance, L. K. Lozina-Lozinskiy subjected the corn borer to freezing and established the extraordinary resistance of these larvae. Larvae, frozen at  $-11.2^{\circ}$  and remaining in this condition from several minutes to 35 days, recovered, the number of those that perished being 36.4%. Under the influence of a temperature of  $-21.2^{\circ}$  for 20 hours, 57.2% of the larvae recovered. Even a temperature of  $-78.5^{\circ}$  lasting from 15 to 20 minutes caused the death of only a certain number of the larvae.

These data cannot fail to indicate the extremely great diversity in the biological properties of insects among which we find individuals who can resist extremely low temperatures. In addition, as we see from the experiments of N. I. Kalabukhov and N. I. Sakharov, in questions of the resistance of insects to low temperatures an important role is played by such factors of nutrition as the amount of water and fatty substances in the insect's body.

With regard to the problem of the anabiosis of microorganisms during freezing we must point out the number of studies of Russian authors on the conditions of microbes in the soils and ice of the permafrost zone as well as under experimental low temperature conditions.

In 1911 V. L. Omelyanskiy recovered microbes from the mucus of the trunk of the Sangayurakh mammoth which was found in the ground in the permafrost zone of Siberia. In 1912, while examining soil samples of the permafrost zone in Amur Oblast taken from a depth of 1.6 and 4 m, B. L. Isachenko detected in the majority of samples sporogenous microbes capable of propagation when placed in a nutritive medium.

These reports and a number of others on the discovery of microbes and certain other very simple animals, such as ciliophora, rotifers, and nematodes, frozen into ice and retaining viability have aroused considerable interest. In this connection investigators raised the question whether life could be preserved in microbes and some other simple animals, frozen into the ice many thousands of years ago and now deep under the surface of the earth in the permafrost zone.

— In 1936 P. N. Kapterev pointed out numbers of plant and animal organisms recovered from the soils and ice of the permafrost zone. In Amur Kray P. N. Kapterev took soil samples from a depth of 2 to 7m in the permafrost zone where the earth was frozen argillaceous soils with strata of ice. The soil samples were carefully collected in order to avoid the entry of embryos of organisms from without. After the samples were thawed out he obtained various species of algae and sporogenous microbes. The results of P. N. Kapterev's research caused doubts among some investigators that methods of soil sampling from great depths were perfected enough to prevent the entry of embryos of organisms from without. However, obtaining various species of algae from various depths suggests that the latter were actually recovered from the permafrost soil.

A. Yegorova isolated sporogenous bacteria and yeasts while examining samples of "stone ice" from the island of Lyakhovskoye.

Considerable work on the study of permafrost zone soils was done by A. Ye. Kriss (1940), who obtained somewhat different data. For instance, while examining permafrost soils from the island of Kolyuchin the author recovered through seeding on special nutritive media (soil agar and meat-peptone-agar) some sporogenous and nonsporogenous bacteria and actinomycetes. Almost identical microflora (sporogenous and nonsporogenous microbes, actinomycetes, and fungi) but in a larger amount were recovered from soil samples from the active layer, that is, the thawing layer above the permafrost strata. In examining soil samples from a depth of 1-1.5 m in a buried peat bog from the permafrost zone of Wrangel Island, cultures were found of white, yellow, and yellow-green micrococci. In the other samples obtained from great depths no microflora were detected.

As the result of his research A. Ye. Kriss believes that only a relatively small number of the various representatives of the microorganisms are able to preserve their viability in the permafrost layers of buried peat bogs. However it is hardly possible that such a diverse number of microbe representatives should retain their viability in a state of anabiosis as indicated by P. N. Kapterev. In A. Ye. Kriss' opinion, the great number of microorganisms and the diversity of species found in the permafrost layer by P. N. Kapterev must be related to the possibility of the entry of the microbes into this layer from the active layer.

In a later work (1944) A. Ye. Kriss and N. A. Grave reported on the results of examinations of ice samples from the permafrost zone of central Yakutia. Examinations were made of an ice sample

in the form of a monolith obtained from a depth of 9 m. The authors assign the formation of this ice to the glacial epoch in Siberia, with the absolute age being of necessity tens of thousands of years old. The monolithic ice sample was very carefully examined at the Permafrost Institute and no viable microorganisms were detected. In the author's opinion, this does not confirm the hypothesis of the anabiosis of microorganisms in glaciers of the permafrost zone.

A. V. Kalyayev (1947) examined samples from permafrost soil at different depths, 4.5, 6.5, 8, 37, 43, 45, and 57 m. After the samples were treated by singeing and removal of the outer layers with a red hot knife, a sterile pincers was used to remove from the center a piece of earth weighing 2-3 g which was again singed and placed into a nutritive medium of MPB (meat-peptone-broth) and MPA (meat-peptone-agar). The experiments were carried out in a laboratory at the very spot where the samples were obtained. As a result of the ground samples obtained from the greatest depths of the permafrost layer, isolated cells (from 5 to 10 per g of earth) of sporogenous aerobic microbes of the genus *Bacillus* were obtained.

From the soil samples taken from lesser depths in the permafrost zone (4 and 6.8 m) nonsporogenous bacteria, cocci and sarcinae, were sometimes obtained.

From the preceding data it is evident that A. V. Kalyayev established the presence of viable sporogenous and nonsporogenous bacteria in soil samples from the permafrost zone. It must be pointed out here that nonsporogenous microflora were recovered only from samples taken from the lesser depths. This situation may be explained by the possibility of later penetration of this

microflora from the active layers of the soil. However we must assume that the presence of nonsporogenous bacteria in the upper layers of the permafrost earth had lasted for a rather long time. Examination of soil samples taken from greater depths of the frozen earth showed that only sporogenous microbes are able to retain their viability at this level.

Thus A. V. Kalyayev's studies of soil samples from the permafrost layer indicate that not only sporogenous but nonsporogenous microbes may remain in a state of anabiosis for a long time but that the sporogenous microbes are more resistant to the action of low temperature.

Butyagin (1909) froze several species of microbes, pathogenic for man, under winter conditions at a temperature of  $-20^{\circ}$  to  $44.8^{\circ}$  and determined that the majority of them retained their viability for 3 months. In addition, it was observed in experiments conducted on alternating cold and thawing that some microbe cultures survived up to 12 freezings and thaws.

B. S. Alejev (1944) points out that many species of microorganisms possess a very high cold resistance.

In some of his experiments microorganisms resisted temperatures near to absolute zero. For instance, bacterial spores germinated following 10 hours in liquid hydrogen at a temperature of  $-252^{\circ}$ . Nonsporogenous microorganisms of the enteric typhoid group were capable of propagation following 20 hours in liquid air at a temperature of  $-172^{\circ}$ .

The results of experiments of a number of investigators testify to the exceptional resistance to low temperature of many

microorganisms. Cultures of the typhus bacillus, *Escherichia coli*, diphtheria bacillus, the bacillus of Asiatic cholera, *Proteus*, lactic acid bacteria, the bacillus of malignant anthrax with spores, and staphylococcus were frozen on gelatin, agar-agar, potato, and in bouillon at a temperature of  $-182^{\circ}$  for 20 hours. After thawing all the microbic cultures were viable and retained their biological properties.

On the effect of low temperatures on the viability of microbes pathogenic for man we have available much information in the literature. However, as a rule, these reports treat merely the transient effect of low temperatures lasting only a few hours. For instance, in developing methods for desiccating microorganisms Soviet investigators (S. S. Rechmenskiy, K. Ye. Dolinov, and L. B. Balayan, A. Klimentova, R. Kruk and G. Yarmol'chuk, D. F. Fedorov, A. A. Batyрева and O. N. Kocher'yan, etc) have studied the action of low temperatures on microorganisms. It has been established that short term action of low temperatures (from  $-20^{\circ}$  to  $-180^{\circ}$ ) had no pronounced injurious effect on the viability of microorganisms. In its results a temperature of  $-20^{\circ}$  was in no way distinguishable from the effect produced at  $-70^{\circ}$  or  $-180^{\circ}$ .

Similar observations of both Soviet and foreign investigators were made in the majority of cases in connection with prefreezing cultures for desiccation. For this reason we made a special study of this problem under experimental conditions.

In veterinary manuals on microbiology, epizootology, and disinfection the effect of low temperatures on pathogenic microorganisms is barely treated. However, this feature is of great importance in studying the epizootic situation for controlling infectious diseases of farm animals. Only with relation to specific

microorganisms, such as those of malignant anthrax, brucellosis, tuberculosis, and certain mold fungi, is it possible to find isolated bits of information on their capacity for resisting the effect of low temperatures to one degree or other (S. N. Vyshel'skiy, N. A. Mikhin, and N. I. Leonov).

In experiments on the long term action of cold on the viability of microorganisms we used a temperature of  $-17^{\circ}$  or  $-20^{\circ}$ . These values were selected as the average extreme temperature observed in the majority of rayons of the USSR. We were planning to determine the resistance of certain microorganisms with relation to the environment of the farm. In addition, we proposed to study the factors making up the action of cold on the viability of microbial cultures during desiccation.

In our experiments we used an electric refrigerator in which the microbial cultures were kept for the required period of time at a temperature of from  $-17^{\circ}$  to  $-20^{\circ}$ . The microbial cultures in the form of bouillon or agar propagations with gelatin or serum added as a protective medium were subjected to the action of the temperature indicated. By this means we proposed to bring our experiments, even though to a relative degree, closer to the natural environmental conditions under which the microorganisms might be subject to low temperatures. In this case we could assume that the microbes in livestock shelters or in the soil following excretion from sick animals were to some degree or other connected with protein substances as products of the decomposition of organic substances. In our control tests the microbial cultures were subjected to the action of cold with protein substances by adding them to a physiological saline solution.

Experimental and control cultures were placed in the condenser of a refrigerator where the temperature could be kept at a strictly controlled level by means of a thermocouple.

In the first experiments the microbial cultures were first covered with gelatin or a physiological solution and then placed in 0.5 ml batches in test tubes and sealed in with plugs of absorbent cotton. After this the test tubes were placed in the refrigerator at a temperature of from  $-17^{\circ}$  to  $-20^{\circ}$ . During the experiment it was noticed that the cotton plug froze through and thus did not prevent the entry of water vapors which condensed on the inner walls of the test tubes in the form of ice. For this reason in following experiments the ampules with cultures were flame-sealed.

The viability of the microorganisms was tested by seeding them onto appropriate culture media at various times, 15, 30, 60, 90, 120, 150, 180, and 210 days. The growth of the cultures after a period in the thermostat was computed for the usual periods, 1, 2, and 3 days. In case of necessity the seedings of the cultures were kept in the thermostat up to 10 days. The results of these experiments are reproduced in Table 1.

TABLE 1

INTENSITY OF GROWTH OF MICROBIAL CULTURES SUBJECT TO THE ACTION OF  
LOW TEMPERATURES  
(from -17° to -20°)

Name of Culture	Strain No	Freezing Medium	Test Time (in Days)							
			15	30	60	90	120	150	180	210
Salmonella enteriditis	315/9	physiological solution	+++	+++	+++	+	-	-	-	-
		gelatin	+++	+++	+++	++	+			
		serum	+++	+++	+++	+++	+++	+++	+++	+++
Salmonella enteriditis	7/1	serum	+++	+++	+++	+++	+++	+++	+++	+++
Salmonella cholerae-suis	133	serum	+++	+++	+++	+++	+++	+++	+++	+++
Malignant anthrax culture	1	physiological solution	+++	+++	+++	+++	+++	-	-	-
		gelatin	+++	+++	+++	+++	+++	-	-	-
		serum	+++	+++	+++	+++	+++	+++	++	+
Brucella	1	physiological solution	+++	+++	+++	+	+	-	-	-
		gelatin	+++	+++	+++	+	+	+	-	-
		serum	+++	+++	+++	+++	+++	+++	+++	+
Streptococcus equi	D 9/1	physiological solution	+++	+++	+++	+++	+	-	-	-
		gelatin	+++	+++	+++	+++	+	+	-	-
		serum	+++	+++	+++	+++	+++	+++	+++	+++
Sheep septicemia culture	24	physiological solution	+++	++	-	-	-	-	-	-
		gelatin	+++	+	-	-	-	-	-	-
		serum	+++	+++	+++	+++	+++	+	+	+
Swine septicemia culture	252	physiological solution	+++	-	-	-	-	-	-	-
		gelatin	+++	-	-	-	-	-	-	-
		serum	+++	+++	+++	+++	+++	+	+	+

Symbols: +++ intense growth

++ growth of medium intensity

+ weak growth

- no growth

From the data in Table 1 we can see that the greatest resistance to low temperature was found in the paratyphoid and brucellosis bacteria and in the bacterial pathogen of malignant anthrax (vegetative form) and Streptococcus equi. The least resistance to cold was found in bacteria of the Pasteurella group.

However when normal serum was added as a protective medium the resistance of these bacteria increased several times. This situation was confirmed by special experiments testing the action of low temperature from  $-17^{\circ}$  to  $-20^{\circ}$  on the viability of microbial cultures by periodic freezing and thawing. These experiments were conducted in connection with the statement of several investigators regarding the destructive effect of low temperature on the viability of microbes where freezing alternates with periodic thawing.

In these experiments we added normal horse serum to the cultures and a physiological solution (1:1) to the control samples. The microbial suspensions thus prepared were dosed out at the rate of 1 ml per test tube. The tubes were flame-sealed and placed under low temperature. The intervals when the action of low temperature was in effect amounted to 2-3 days while the thawing periods were 2 hours long.

TABLE 2

EFFECT OF LOW TEMPERATURE ON THE INTENSITY OF GROWTH OF BACTERIAL  
CULTURES IN CASES OF PERIODIC THAWING

Name of Culture	Strain No	Freezing Medium	1	2	3	4	5	6	7	8	9	10
Salmonella enteriditis	315/9	physiological	+++	+++	+++	+++	+++	+	-	-	-	-
		solution	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
		serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Salmonella cholerae-suis	133	physiological	+++	+++	+++	++	+	+	-	-	-	-
		solution	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
		serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Salmonella cholerae-suis	1	physiological	+++	+++	+++	+++	++	++	-	-	-	-
		solution	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
		serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Streptococcus equi D9/1		physiological	+++	+++	+++	+++	+++	+	-	-	-	-
		solution	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		serum	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sheep septicemia culture	24	physiological	+++	+	+	-	-	-	-	-	-	-
		solution	+++	+++	+++	+++	++	-	-	-	-	-
		serum	+++	+++	+++	+++	+++	++	+	-	-	-
Swine septicemia culture	25	physiological	+++	+	+	+	-	-	-	-	-	-
		solution	+++	+++	+++	+++	+++	++	+	-	-	-
		serum	+++	+++	+++	+++	+++	++	+	-	-	-
Ditto	252	physiological	+++	+	+	-	-	-	-	-	-	-
		solution	+++	+++	+++	+++	+++	+	+	-	-	-
		serum	+++	+++	+++	+++	+++	+	+	-	-	-

Symbols: +++ intense growth

++ growth of medium intensity

+ weak growth

- no growth

From the data cited in Table 2 we can see that repeated freezing and thawing did not have a very harmful effect on the viability of microorganisms. Only Pasteurella bacteria, to which a physiological solution had been added, lost their viability following 3 freezings and thawings. Following the addition of serum

the Pasteurella cultures acquired marked resistance to cold and resisted repeated (no less than 7) freezing and thawing. As for the bacterial cultures of Streptococcus equi and paratyphoid fever in calves and pigs, they had exceptional resistance to low temperature with repeated freezing and thawing. For instance, these microorganisms retained viability when frozen and thawed 6 times when added to a physiological solution and 10 times when added to serum. Obviously these results do not represent a limit for these microbes since culture growth was still quite intense when the investigations were terminated for technological reasons.

Test experiments with the cultures of bacteria of paratyphoid fever of calves and Streptococcus equi in a serum medium showed that they retained their virulent properties following prolonged freezing. These same cultures lost their virulence for white mice to a great degree following repeated freezing and thawing although a portion of the mice died when inoculated with large doses (50-100 million bacterial cells).

Thus the experiments which we carried out showed that in taking measures to control brucellosis, paratyphoid fever, and streptococcal and pasteurellar infections the great resistance of these pathogens to low temperatures must be taken into consideration. Specific prophylactic measures against animal infection and the disinfection of buildings must be carried out not haphazardly but systematically at any time of the year depending on the appearance of the infection. In addition, there is apparently great resistance to cold among other pathogens as confirmed by experiments in freeze-drying a large number of different microorganisms, pathogenic for man and animals, as well as by the prolonged life of sporogenous and nonsporogenous microbes in soils of the permafrost zone.

Our experiments gave us the opportunity to study only the resistance of microorganisms to low temperatures without taking into consideration the dying of the bacterial cells during the freezing process. With this aim we set up special experiments to study the action of low temperatures of  $-17^{\circ}$  and  $-20^{\circ}$  on the vegetative form of the malignant anthrax pathogen. A bouillon culture of the anthrax pathogen cultured for 12 hours in a thermostat was transferred onto meat-peptone-agar and cultured for 10 hours. The culture was washed with a physiological saline solution and tested in a diluted drop and in a strained smear for the absence of spore formation.

After establishing the optical concentration according to the bacterial standard of 500 million bacterial cells per ml the culture of malignant anthrax pathogen was diluted to 1:1 in one case with a physiological solution and in another with a 10% solution of gelatin. Both dilutions were poured into test tubes (0.5 ml each) and frozen at  $-17^{\circ}$  and  $-20^{\circ}$ .

Before freezing began, the number of viable anthrax bacilli was determined in each dilution by transference to meat-peptone-agar in bacteriological dishes. Analogous investigations were made after the test tubes had been in a refrigerator 2 hours and then again after 1, 5, 20, 40, and 60 days. As a result it was established that after 2 hours of low temperature the number of living bacterial cells is 61.8% in the gelatin culture. After 24 hours the number of viable cells had dropped to 37.2%. A further decline continued gradually and reached 4.5% by the sixtieth day. In the culture without gelatin the dying process took place much more rapidly and no growth of anthrax culture was observed on the sixtieth day when a culture medium was seeded with the final dilution.

From the data cited it is clear that the dying process among microorganisms takes place under low temperatures with a greater intensity during the first hours than during the subsequent period. The use of gelatin as a protective medium is very effective. While the number of viable bacterial anthrax cells in a gelatin culture was 61.8% following 2 hours in the cold, 37.2% after 24 hours, 25.6% after 5 days, 23.2% after 20 days, 14.5% after 40 days, and 4.5% after 60 days, the dying process was much more rapid in the cultures without gelatin. There the number of viable cells in a physiological solution was 32.7% after 2 hours of cold, 18.4% after 24 hours, 7.6% after 5 days, 4.5% after 20 days, 3.0% after 40 days, and after 60 days there was no growth at all (Figure 21).

When an analogous experiment was set up with other strains of the anthrax pathogen in a serum medium the culture retained its viability for 200 days. However, the dying process of the bacterial cells proceeded in the same fashion.

Thus our experiments aimed at studying the resistance of microorganisms to low temperatures indicated that microbial cultures in the majority of cases reveal pronounced resistance to cold and are capable of retaining their vital properties for a long time in a frozen state.

In considering the question of the resistance and dying of microorganisms when affected by low temperatures it must be pointed out that the mechanism of this action has until recently not been quite clear.

V. V. Yefrimov (1922), in studying the action of low temperature on infusoria, observed that the destruction of the infusoria

takes place not as the result of ice pressure developing around the infusorial body but as the result of the formation of ice crystals in the colloidal membrane and in the very protoplasm of the cell. In this case the ice crystals extract moisture from the organism of the infusoria which causes irreversible changes in the cells. At the same time he observed that the higher temperatures, near the freezing point, effect the viability of the organisms more strongly than the lower temperatures.

In the works of E. Ya. Grayevskiy (1946-1948) and other investigators it is shown that the retention of viability in bacterial and plant cells is caused by the presence or absence of ice crystals in them during freezing.

The so-called theory of vitrification of living cells served as the basis for explanations of this type. The authors believe that under the action of low temperatures ( $-172^{\circ}$ ) the solidification of the protoplasm of living cells sets in suddenly, thanks to which the liquid substances in the cells and the liquid of the surrounding medium pass into a vitreous state, by-passing the phase of crystallization. When higher temperatures are used in freezing the solidification of the cellular protoplasm takes place more slowly in view of which the crystals which cause their destruction are formed in the cells and in the surrounding liquid.

E. Ya. Grayevskiy used liquid air on amebae, spermatozoa of frogs, spermatozoa of rats and dogs, unicellular layers of epidermis from an onion skin, muscle fibers of a frog, and certain microbial cultures. Positive results of resuscitation were obtained in experiments with the spermatozoa of frogs, the onion skin epidermis, and the microbial cultures. In this case great interest is aroused by the results of experiments with microbial cultures since the subject of the chapter at hand is the anabiosis of microorganisms.

From a number of microbial cultures, E. Ya. Grayevskiy subjected strains of typhoid fever, paratyphoid A and B and Breslau culture, dysentery, Escherichia coli, and yeasts to the action of liquid air. As a result it was determined that deep freezing has a different effect on the viability and biological properties of microbial cultures. For instance, in cultures of certain species, yeast, for example, he noticed an abrupt decline in the number of living cells while in other species of bacteria a large part of the cells retained their viability. It was also determined that the number of viable bacteria cells and yeasts following deep freezing fluctuates greatly from experiment to experiment. Despite this fact, the author concludes that deep freezing of microbial cultures has a less disadvantageous effect than higher freezing temperatures although this statement cannot be confirmed by more specific and accurate data. The author believes that it is not the low temperature in itself which causes damage to the cells but the crystallization of frozen water in cells which takes place only at the moment of chilling or thawing.

It must be pointed out that data on the viability and dying of microbial cells acted upon by liquid air, cited by Grayevskiy in his work, still do not indicate the more favorable effect of deep freezing on the viability of microbes. Analogous results are usually obtained when microbial cultures are treated with temperatures of  $-20^{\circ}$  and  $-70^{\circ}$ . As for the extremely great resistance of microbial cells to low temperatures in general, this is apparently related not only to crystallization but to the biological properties of microorganisms as the most minute creatures.

Other investigators, like Grayevskiy, have succeeded in vitrifying the spermatozoa of a frog. Following thawing and warming, approximately 20% of the spermatozoa were viable. Experiments in vitrifying moss leaves were likewise successful.

It must however be pointed out that experiments in vitrification carried out by Grayevskiy and other investigators were much more successful when the spermatozoa of frogs or moss leaves were first more or less dehydrated through the action of strong sugar solutions.

Experiments in vitrification with beer yeasts showed that for a successful transition from the liquid state to the vitreous without going through the crystallization phase the organism must have very small dimensions since in this way there is less possibility for the formation of crystallization centers in it. In order to avoid the formation of crystals in the microbial cell the rapidity with which the low temperature acts on the organism must be extremely great.

When beer yeasts were cooled to low temperatures we used a special method for staining the cells with methylene blue. This method allowed us to determine the amount of dead and viable cells since the first took on a dark stain and could easily be calculated from microphotographs while the others remained unstained.

The authors of these experiments believe that the less the number of cells undergoing deep chilling the less the likelihood of crystals forming in them and vice versa, the greater the number of cells the greater the possibility of the formation of ice crystals.

While this situation does not exclude the theoretical explanation of the causes of the great resistance of plant organisms and microbial cultures to low temperatures as vitrification, it does, however, indicate that in microbial cells the ice crystals apparently do not form, thanks to their small dimensions. As for the great resistance of the microorganisms to deep freezing and certain advantages

of cooling the microbes at low temperatures, this may be explained by the fact that at these temperatures evidence of irregular effect of cold on the microbes and factors of partial defrosting (thawing) are completely eliminated. Partial thawing during freezing has a very unfavorable effect on the organisms. This alone, from our point of view, is the advantage of cooling to very low temperatures as found in experiments. The positive side of low temperatures ( $-172^{\circ}$ ,  $-252^{\circ}$ ) must be included in their use for deep freezing more complex organisms with larger dimensions than the microbial cell. In this case the vitrification factor must be of great importance since the sudden solidification of the body without the crystallization phase prevents the formation of ice crystals which cause the death of the organism.

In reviewing the causes of the resistance to cold in plant cells, microbial cultures, and microscopic animals we must consider the problem of the resistance to cold of spermatozoa of mammals. In this case we must look at the work of I. V. Smirnov (1949) who reported on extremely interesting experiments with the deep freezing of spermatozoa of mammals. The author started with the vitrification theory. He managed to develop a method for preserving all the biological properties of rabbit spermatozoa for a long time (up to 32 days) and in principle solved the problem of preserving spermatozoa of bulls, stallions, and rams in a frozen state.

I. V. Smirnov points out that attempts to preserve the viability of mammalian semen by freezing at  $-6^{\circ}$ ,  $-19^{\circ}$ , and  $-20^{\circ}$  have not been crowned by success. For this reason he believes that the preservation of vital properties of spermatozoa and their capacity for insemination can be achieved only by deep freezing at a temperature of  $-78^{\circ}$  and  $-196^{\circ}$ , since at this temperature the vitreous state

of the spermatozoa is achieved without passing through the crystallization phase. In making experiments to preserve the spermatozoa of rabbits at temperature of  $-78^{\circ}$  and  $-196^{\circ}$  and to use them for the impregnation of does, I. V. Smirnov obtained most encouraging results. For instance, when he inseminated 61 does with spermatozoa kept for various lengths of time (2, 15, 21, and 32 days) under deep freeze conditions, he obtained litters from 39 does with the young rabbits having a satisfactory course of development. The data obtained allowed the author to conclude in favor of the use of deep freezing for the preservation of semen and their use for artificial insemination.

Thus the action of low temperatures on the more complex organisms of the spermatozoa under specific conditions not only does not result in their destruction but contributes to preserving their viable and fertilizing properties for a longer time.

At present the deep freeze method is likewise being used with the aim of preserving the smallest invisible beings, the viruses, for a long time. Although this method is not as much improved as the drying method, it does permit us to preserve the virulent properties of viruses under laboratory conditions.

In analyzing the exceptionally great resistance to low temperatures of microbial cultures, microscopic animals (rotifers, tardigrades, nematodes, infusoria, and others), and the spermatozoa of frogs and rabbits, the question arises as to what are the causes of this resistance.

If we review the problems of anabiosis of living creatures under the action of cold we find it established that in freezing the cells are completely dehydrated with their liquid being transformed into a solid state, that is, ice. If the cooling temperature

is not too low, for example  $-20^{\circ}$  to  $-30^{\circ}$ , the organisms retain their vital properties although greatly retarded, in the opinion of individual investigators (Shmidt, Lozina-Lozinskiy, Sakharov). If the cooling temperature is very low ( $-252^{\circ}$ ,  $-272^{\circ}$ ), approaching absolute zero, certain authors (Preyer, Claude Bernard, P. Yu. Shmidt) believe that cell life comes to a complete halt and anabiosis as conceived by Preyer and P. I. Bakhmet'yev sets in.

However these assertions may not be wholly correct since the resistance of microorganisms and microscopic animals to low temperatures must obviously be determined not by the number of degrees of the freezing temperature but by their biological properties, by the simpler cell structure of the organism, and by their size. This can be confirmed by the results of numerous investigators who have studied the effect of low temperatures on various organisms. For instance, the use of cold in our experiments in freezing cultures of the anthrax pathogen showed that temperatures of  $-17^{\circ}$  and  $-20^{\circ}$  cause the destruction of 38.2% of the microbial cells during the first 2 hours and that the dying of the microbes then proceeds very slowly. If cultures are frozen at  $-17^{\circ}$ ,  $-20^{\circ}$  in a mixture with serum colloid, the viability of the various pathogens of infectious diseases can be preserved for 10 months and longer without diminishing the intensity of growth when transferred to nutritive media.

Unsuccessful attempts at freezing and reviving many of the simplest organisms can apparently be explained by their comparatively complex structure and greater size. In order to overcome this obstacle it is necessary that such organisms be partially dehydrated in advance or treated with a mucilage agent. This is indicated by the most propitious results obtained from using serum colloid in our experiments with microbial cultures and from Grayevskiy's preliminary treatment of frog and rooster spermatozoa with strong salt and sugar solutions before freezing.

### Anabiosis in Desiccation

Many investigations have been made with regard to the phenomenon of anabiosis in the desiccation of microscopic animals, such as inhabitants of the mosses and lichens, insects, amphibians, and some warm blooded animals. Soviet scientists especially have conducted extensive research in this field.

There is no reason for stopping to treat problems of anabiosis in the desiccation of insects, amphibians, or warm blooded animals, since this would take us off into another area and would make more difficult a concrete exposition of the problems of anabiosis in microscopic animals and microorganisms.

As already indicated, the definition given by Preyer (1873) of the condition of dried microscopic animals, that is, rotifers, tardigrades, and nematodes, as anabiotic with total cessation of manifestations of life continued to be contested by many investigators. The author in his work on anabiosis identified 2 contrary forms of the absence of life. The first is when a living creature may be lifeless yet capable of life and the second when a creature is lifeless but incapable of life, that is, dead.

This is the starting point for treating the concepts of anabiosis of organisms during desiccation when the organism is in a lifeless condition but is capable of reviving when environmental conditions are changed. Some other investigators hold to this same point of view.

Ye. A. Shults dried rotifers, tardigrades, and nematodes and established that when water was added the animals revived. The author also studied the question as to whether vital functions cease during desiccation. In order to do this he placed dried organisms for 2 weeks in pure hydrogen and the animals revived after thawing

and hydration. This experiment, in the author's opinion, proves the absence of gas exchange and oxidizing processes in the organism of the dried animals.

A great deal of work on the effect of desiccation on the organisms of rotifers, tardigrades and nematodes was done by P. Yu. Schmidt (1922). He determined that a change in moisture and temperature has a destructive effect on the viability of the organisms when kept in a dry state and a larger number of dried animals die as the storage time is extended.

In order to test the possibility of total desiccation, P. Yu. Schmidt made the following experiments with rotifers. The animals were first dried on covered slides in air and then placed in test tubes with calcium chloride for more complete desiccation. After this the rotifers were transferred to wide tubes containing metallic sodium. For 16 days the air was exhausted from the test tubes by a mercury pump with the vacuum reaching 0.2 mm. Then the test tubes with the rotifers were sealed and stored for 3 months.

This method allowed the investigator to remove traces of moisture from the dry rotifers. The metallic sodium was thereupon converted into sodium hydroxide. In this case oxygen was removed from the test tubes containing the rotifers and only a small amount of nitrogen and hydrogen remained in them. When tested after 3 months a portion of the rotifers revived.

P. Yu. Schmidt believes that with the removal of moisture from the rotifer organism and the absence of oxygen in the test tube respiratory processes in the organism cease. However, objections regarding minimal (retarded) life are not eliminated by these experiments since in the author's opinion this would necessitate subjecting the dried rotifers to the action of very low temperatures.

Experiments in drying rotifers, tardigrades, and nematodes in approximately the same form were carried out in 1923 by other scientists and proof was given of the possibility of these animals' retention of their viable properties for a long time in a dry state. In addition, the dry organisms were subjected to cold by placing them in liquid air at a temperature of  $-180^{\circ}$ , in liquid hydrogen at a temperature of  $-253^{\circ}$  and liquid helium at  $-271^{\circ}$ . The action of liquid air showed that the animals retained their viability at this temperature for 20 months. They did not lose their viability when under the influence of liquid hydrogen for 26 hours nor under liquid helium for 7 hours.

P. Yu. Shmidt in his book, Anabioz [Anabiosis] (1948), in analyzing data from observations of investigators in the field of the anabiosis of rotifers, tardigrades, and nematodes, points out that very retarded life processes may take place in the organism of the dry rotifers, tardigrades, and nematodes only when they are dried under natural conditions where a slight amount of moisture may be retained. Under experimental drying and storage under vacuum the vital process in the organism of these creatures comes to a complete halt. This lifeless state, in P. Yu. Shmidt's opinion, may last for a very long time and after the vacuum is resolved and water is added the life processes of the organism are restored and the animal revives.

As a basis for a theoretical hypothesis on the cessation of life processes when rotifers, tardigrades, and nematodes are desiccated experimentally, P. Yu. Shmidt cites the deep freeze factor. While minimal gas exchange, and consequently, retarded life are theoretically possible in dry organisms that contain a little moisture and are kept under natural conditions, the author says,

with the action of low temperatures of  $-253^{\circ}$  and  $-271^{\circ}$  no life processes or gas exchange in the organism are possible. Hence P. Yu. Schmidt in essence shares the opinion of those who believe that individual living creatures may have a total cessation of life processes during desiccation and may revive following the addition of water.

Becquerel holds approximately the same point of view with regard to anabiosis in the inhabitants of mosses and lichens (rotifers, tardigrades, infusoria, amebae) and certain algae when dried under natural and artificial conditions. For example, he believes that in desiccation these creatures pass from active to retarded life. This retarded life of individual organisms in a dry state under vacuum may last not for months but for years. For instance, cultures of algae dried by Becquerel and kept in a dry state under vacuum for 22 years were viable when examined. At the same time he believes that certain organisms, and algae in particular, when desiccated under vacuum and cold for 20 hours in liquid nitrogen and for 7.5-16 hours in liquid helium, pass into a "state of the absence of life," that is, into a state of anabiosis in Preyer's meaning of the word.

Thus the basis for adherents of the theory of total cessation of life processes in the dry organism of microscopic animals is the criterion of the capacity of the organism to resist deep freezing in a dry state. However there is hardly any difference in the effect on the body of almost total desiccation without oxygen and deep freezing. If we approach this question from the point of view of the presence or absence of the metabolic process in the organism, they must in a like degree be reduced to the minimum or to total cessation. In addition, the action of deep freezing on the organism

already in a dry state cannot be stronger if only for the reason that the organism in this case contains practically no water and is extremely resistant to the action of environmental conditions, including deep freezing and heating. In addition, we can hardly distinguish the effect of a temperature of  $-75^{\circ}$  or  $-180^{\circ}$  on the organism in a dry state from that of deep-freeze temperature ( $-253^{\circ}$ ,  $-271^{\circ}$ ).

It seems to us that the criterion of the cessation of living processes in the organisms of rotifers, tardigrades, and nematodes is not the factor of the extreme deep cooling but the factor of the absence of metabolism in the organisms of these animals. We still do not know a situation where metabolism would be possible in even a retarded form when organisms are kept for a very long time in a dry state under vacuum.

While treating the adherents of the idea of minimal life in organisms when desiccated, we must point out the work of certain investigators. Some of them, for example, believe that desiccation, like low temperatures, creates conditions under which all chemical reactions are impeded, thanks to which there is less destruction in the rotifer organism. The authors express the conjecture that the drying of the rotifers is apparently not absolute, that some traces of moisture remain in the body and this is sufficient for the prolongation of life. As a result of observations the investigators come to the conclusion that there is no basis for assuming a total cessation of metabolism in the body of the dried rotifers. While there is a trace of moisture, life processes are possible although obviously in a very retarded form. Thus the authors believe that rotifers in a dry state have a latent and retarded life.

In his book on Spyachka zhivotnykh [Dormancy in Animals] (1936), N. I. Kalabukhov points to the possibility of life processes and consequently of retarded metabolism in the organism of dried rotifers, tardigrades, and nematodes subjected to desiccation under natural conditions. In addition, the author believes that life processes are possible even under artificial desiccation if a certain amount of moisture is retained in the body. For this reason the author expresses doubts as to the total cessation of life, since the possibility of reviving dried animals is limited to a specific time, that is, depends wholly on the length of the storage period in a dry state.

On the problem of anabiosis in tardigrades during desiccation we have the report of N. F. Labunets (1950) in which the author describes his experiments using the in vivo method of staining reserve cells with neutral red in order to elucidate the phenomenon of desiccation of animals.

The fact is that in the cavity of the tardigrades there is a reserve substance called the reserve cells. The latter contain a nutritive substance and during hunger the number of these cells decreases in view of the consumption of the nutritive material bound in them.

Having set himself the job of studying the condition of the tardigrade organism in the dry state, N. F. Labunets quite correctly turned his attention to the problem of the possibility of fixing these cells in order to determine the presence or absence of life processes in the organisms of these animals. For this purpose he proposed a special method for staining the reserve cells of tardigrades red in vivo by using neutral red. When the tardigrades are placed in the stain solution the first to be stained are the

reserve cells of the throat, then of the entire intestinal tract (the stain penetrates only through the intestine but not through the cuticle). After 2 days the tardigrades were removed from the stain solution, the number of stained reserve cells was computed and a sketch was made of the body of each tardigrade (Figure 22). After this the stained tardigrades were dried on object glasses in a desiccator over calcium chloride at a temperature of  $14^{\circ}$ ,  $15^{\circ}$  for different periods of time (from 10 to 60 days) and then hydrated for the purpose of total restoration of life processes.

As a result of the experiments done by N. F. Labunets he determined that in the organisms of revived tardigrades there had taken place grave modifications in the sense of a reduction in the number of reserve cells.

The diminution in the number of reserve cells in tardigrades during the period in the desiccator shows that vital processes in the organism do not cease during desiccation since the dying of the reserve cells indicates the consumption of the accumulated nutritive material found in them.

Thus the theoretical hypotheses of a number of investigators on the retarded (minimal) life for a short period of time in the organism of microscopic animals such as the tardigrade, rotifer, and nematode in a dry state under natural conditions were proven by the experiments of N. F. Labunets.

However these experiments must not be considered as proof in explaining the vital processes in the organism of tardigrades kept for a long time in a dry state under vacuum. Labunets used a simple drying method without using a deep vacuum and his observations of the dried animals were made during a very limited period

of time (10-60 days). In addition, inasmuch as the tardigrades were dried in a desiccator without a deep vacuum, the degree of desiccation was obviously not thorough which might cause a gradual dying of the reserve cells as the tardigrades dried out. Labunets' experiment is closer to natural conditions of desiccation of the tardigrades in mosses and lichens than to experimental desiccation in a vacuum apparatus with subsequent long storage under vacuum.

While the theoretical basis of the vital processes in rotifers, tardigrades, and nematodes during desiccation presented very great difficulties, the problem of manifestations of life when minute creatures like the microorganisms are dried is difficult to an even greater degree.

The study of manifestations of anabiosis in rotifers, tardigrades, and nematodes in the dry state has permitted investigators to penetrate more deeply into the essence of the biological properties of these animals. Their comparatively great size makes it easier to observe each animal apart under normal conditions under the microscope, in a dry state, and during the restoration of vital processes, that is, during the transition from the dry to the original state. Making such observations during the study of dry microbic cultures has been hampered greatly by the extremely small size of the microbial cells.

In our work on the desiccation of microorganisms we aimed at studying the manifestations of life in microbes in a dry state. As a basis for our research we selected the well known tenet of I. V. Michurin and T. D. Lysenko regarding the role of the environment and its effect on the organism. By changing the environment of the microbes during desiccation we basically changed their metabolism.

As a consequence of the radical change in the environment the viability of the microbes was not lost but was succeeded, apparently, by a temporary cessation in the vital processes.

Before passing to a survey of the question of anabiosis in microorganisms during desiccation, let us deal briefly with characteristics of nutrition and respiration in microbes under ordinary condition of cultivation.

As we know, a constant metabolic exchange between the cell and the external environment is a necessary condition for the life of the microbes. The microbes assimilate and excrete various gases and liquid substances during metabolism. In the microbial cell numerous complex processes of assimilation and dissimilation are constantly taking place. The nutrition of the microbes demands nitrogenous substances, carbohydrates, and a small amount of the minerals. The nitrogenous substances are used up in building the body of the microbial cell while the nonnitrogenous substances (carbohydrates) serve as a source of energy.

The process of microbial nutrition is accompanied by a destruction of substances and their conversion into new compounds. The destruction of various substances in the microbial cell is in turn accompanied by exothermic reactions which constitute the essential feature of respiration for a large number of microbes. Thus during metabolism the microbes are in constant need of nutritive substances and free energy.

The processes of nutrition and respiration are constant in the microbial cells and the more energy involved in these metabolic processes the more intensive the developmental activity, that is, propagation, aging, and dying. The termination of these processes first brings the microbe to retarded life and then to death.

Among the elements necessary for microbe nutrition, the principal place is occupied by the organogens C, H, and O from which protein, the principal life bearer, is constructed. Microbe nutrition takes place under conditions of specific humidity when the nutritive substances are capable of passing through the membrane of the microbial cell. For this reason microbial cells contain an average of 80% water. Studies have shown that the water contained in any nutritive substrate where microbes propagate has a marked influence on the entire course of their development. From this it is clear that nutritive substances dissolved in water are one of the essential conditions under which the nutrition of bacteria takes place.

All the characteristics of the cells, that is, the capacity to reproduce and the capacity for motion and metabolism depend basically on the presence of moisture in the external environment and in the bacterial cells themselves. An increase or decrease in the amount of water in the living cell under normal conditions is reflected very strongly in its viability. The absence or insufficiency of moisture in microbial cells may induce a cessation of all vital functions and even death.

A second basic condition for the vital processes of bacteria is respiration. Facultative anaerobes absorb atmospheric oxygen and secrete carbon dioxide and water as end products of respiratory exchange. Many species of microbes possess an incomplete respiration where the processes of oxidation of organic substances does not carry through to the formation of carbon dioxide and water as end products of oxidation.

The respiration process in anaerobic bacteria takes place not through oxidation but through the splitting of organic compounds in

the absence of free atmospheric oxygen. For this purpose the exothermic reaction may be utilized, characterized by the liberation of energy independently of whether it is oxidation or reduction. However, certain anaerobic bacteria may develop under environmental conditions where there is a slight amount of oxygen. In addition, anaerobic microorganisms utilize for their development the oxygen of nutritive media where it is bound with organic compounds.

In general, independently of the nature of respiration in various microbes when stored under ordinary conditions they need certain conditions, that is, the presence of a liquid medium.

Otherwise the process of gas exchange in microbes must inevitably come to a halt which in turn induces a termination in metabolism.

As we see from the above, microbes require nutritive substances, water, and atmospheric oxygen for life and development. For a maximal retardation of the life processes in microbial cells, for cessation, that is, where the cell is in a state of anabiosis, the microbes must be deprived of moisture and atmospheric oxygen. These conditions are created when microbial cultures are desiccated and stored under vacuum. However, it is not enough simply to transfer the microorganisms from one state to another.

In order to achieve the preservation of a significant number of viable individuals and their basic biological properties we need to create a special defensive medium based on the biological properties of the microbes.

Thus, in the light of present day knowledge of the problem of desiccation of biological substances and living creatures we need not only to develop new methods for preserving the cultural

biochemical and virulent properties of microorganisms but to find a theoretical basis for the possibility of prolonging the life of microbes under new and artificially created conditions.

The complexity of determining the state of such microscopically small creatures as microbial cells in the dry stage limited us in our study of this problem with relation to the use of already known methods. For instance, it was impossible to use the microscope to study the visible condition of microbial cells (stage of desiccation and revival following addition of water). In this case, when studying dry cultures, we had to employ methods based on maximal dehydration, the storage of the cultures under vacuum, and a study of the viability of the microbes by determining the number of living microbial cells after a specific storage period.

The method of desiccating microbial cells from the frozen state under vacuum has been widely used in medical and veterinary institutions. In works published on this problem we have had by and large a description of the technology of desiccation and the use of dry microbial cultures. However there are as yet no exhaustive explanations of the exceptional resistance to the influence of the environment which is acquired by microbial cells when desiccated artificially.

As already pointed out, while the preservation of life in microbes under ordinary conditions requires certain conditions such as the presence of moisture, nutritive substances, and atmospheric oxygen, the preservation of viability in desiccated microbes requires a directly opposing environment. In this we must consider the dialectical opposition when negative acting causes (desiccation) are converted into positive ones where there is an abrupt change in the environment of the microorganisms.

However, it would be incorrect to believe that desiccation alone is sufficient for this. In order to preserve life as long as possible in dry cultures, many investigators have spent a lot of time seeking additional substances which aid in protecting the microbial cells during experimental desiccation and during preservation. Such protective substances are the protein colloids and certain crystalloids. In addition deep vacuum must be added to the number of defensive factors.

In order to change the character of the life of microbes it was necessary to create conditions under which there would not be a termination of life, that is, death, but at the same time there would not be intensive life processes in the microbial cells, since the more intense the vital processes, metabolic processes, developmental processes, the more rapid the aging and dying of the microbial cells. For instance, we know that the average rate of propagation of microbial cells in the majority of bacterial species is approximately 70 generations per day, that is, cell division takes place each 17-25 minutes. In this process a part of the microbes soon start to die while another portion of the cells continues to develop and produce new generations of cells, etc. Hence it follows in theory that the more retarded the vital processes in microbial cells, the longer the viability of the cells should be retained.

With regard to explaining the retarded life processes in certain microscopic animals and microorganisms under natural conditions it is essential to point out manifestations of retarded life in certain more highly organized animals which go into periodic hibernation. During this time the nourishment of the animals from without comes to an end and the vital processes in

the body take place much more slowly. Almost the same thing can be observed in the inhabitants of the mosses and lichens when the organisms of rotifers, tardigrades, and nematodes and certain other animals which become encysted under natural conditions are capable during desiccation of passing into an anabiotic state and preserving their vital properties for a long time. The vital processes in these dried animals are very slow, in the opinion of the majority of investigators.

Among the microorganisms capable of undergoing desiccation under natural conditions must be included the sporogenous microbes of the soil. During their evolutionary development the latter worked out a defense reaction against unfavorable influences on the part of the environment. This reaction is characterized by a large loss of water in the cells, by intensive thickening of the protoplasm, and the formation of an almost impenetrable membrane around the spore. We suppose that the vital processes in these spores is greatly retarded, that is, they are in a state of anabiosis.

If we analyze all that we have said about the anabiosis of microscopic animals and sporogenous microbes under natural conditions, we may draw the following conclusions.

By experimentally changing environmental conditions of non-sporogenous labile microorganisms, we may induce in them a state of anabiosis. For this reason the work of many investigators in the field of desiccation of various cultures has been characterized by a tendency toward the creation of new conditions for the microorganisms and viruses.

In studying the length of time during which dry microbial cultures can retain their viability some authors (Dolinov and Balayan, Kolesov, Kozlov, Del'nik and Vinogradov) tested not only

their vegetative biochemical and virulent properties but took into consideration the number of viable and dying microbial cells. As a result of the research it has been established that the vital properties of dry microbial cells are preserved for a very long time.

For instance, K. Ye. Dolinov and L. B. Balayan (1946) investigated strains of dry pneumococcus culture in storage for 38 months and established the gradual dying of some microbial cells. The authors point out that the number of viable microbes following desiccation and preservation under vacuum for 38 months was reduced in one case to 33 and in another to 79%. It is characteristic that the viable properties of pneumococcal microbes are retained much better following desiccation in serum. For instance, the number of viable pneumococcal cells dried in serum was 33-79% when tested after 38 months, while in a desiccated bouillon culture the number of viable cells reached only 2.8-8%. On this basis the authors have concluded that the gradual dying of microorganisms during their preservation in a dry state shows that the vital processes in the cell do not cease.

Yu. A. Kozlov, B. S. Del'nik, and V. V. Vinogradov (1948) dried bacterial cultures of typhoid fever, paratyphoid A and B, dysentery, brucellosis, staphylococcus, Escherichia coli, and others. For a drying medium they used a 10% solution of sucrose with 1.5% of gelatin. As a result of the examination it was found that after a year's preservation the dried microbial cultures contained 1-7% viable cells. The slight percentage of viable cells was regarded by the authors as a feature of the methodology employed and as due to a slight trace of moisture in the cultures following desiccation. However, this method, in the authors' opinions, is

thoroughly useful for preserving museum cultures. Concerning the condition of the microbes, the authors indicate that the life processes in dry cultures do not come to a halt and that lysis of bacteria takes place during the entire preservation period under vacuum and is even quite intense in the more labile cultures.

In our experiments (1944-1948) we dried bacterial cultures of calf and pig paratyphoid fever, brucellosis, pasteurellosis, lamb dysentery, strangles streptococci, swine erysipelas, glanders, and pullorum disease of poultry. A more detailed exposition of the results of these experiments will be given in a special chapter. Here we want only to mention the problems of viability of dry microbial cultures and their resistance to external factors.

Many investigators (Dolinov and Balayan, Kozlov, Del'nik and Vinogradov) have established the fact that as the result of the desiccation of microbial cultures in a frozen state a certain number of the microorganisms die. However scientific literature has as yet presented no data on the causes of death among the microbial cells. We do not know whether this takes place as a consequence of the action of low temperature or if the desiccation process itself has an unfavorable effect on the individual microbes. For this reason we thought it necessary to study the effect of each fact by itself, in order to get a fuller concept of the causes of death in microbial cells following freezing, desiccation, and storage. With this aim in mind we set up special experiments using a culture of the calf paratyphoid fever pathogen.

The order of the experiments was as follows. After washing and determining the optical concentration in a paratyphoid culture we determined the number of viable microbial cells. Then we added

to the culture as a protective medium normal serum in one case, gelatin in a second, and a physiological saline solution in a third. The culture samples thus prepared were packed in specific doses in ampules and first frozen and then dried.

Determining the number of viable cells indicated that a large percentage of the microbes die during freezing and a smaller number during desiccation. For instance, in the serum culture the number of viable microbial cells following freezing was 60%, following desiccation 48%. In the gelatin culture the number of viable cells was 55.1% following freezing, 44% following desiccation. In the culture with the physiological saline solution the number of microbial cells after freezing was 13.6% and after desiccation 7.3% (Figure 23).

Experiments showed that the majority of the microbial cells die as a result of the action of cold and not as the result of the desiccation process and that the protein colloid used acts as a great protective agent for the microbial cells during freezing and desiccation.

In order to study the condition of the dried microbial cultures during prolonged preservation we set up experiments for determining the number of viable cells. In this case the experiment was made with the paratyphoid culture described above. The culture was kept under vacuum in ampules at ordinary room temperatures of 15-20° and 27°. In the culture dried with serum there was 1.0-1.3% of residual moisture, in the gelatin culture 3.4%, and in the culture with no protein colloid 0.8-1.2%.

When the experiments were carried out we obtained the following results. In the serum culture after 12 months of storage the number of viable microbial cells was 41.8%, after 24 months 34.5%,

and after 36 months 30.4%. In the gelatin culture the percentage of viable microbes was 38.7 after 12 months, 13 after 24 months, with no growth at all after 36 months. In the culture to which no protein colloid had been added the number of viable microbial cells was 4.5% after 12 months, 3.7% after 24 months, and there was no growth at all after 36 months (Figure 23).

From the data cited from the study of bacterial cultures of the pathogen of paratyphoid fever in calves we can see that the viable properties of microbes are not lost during desiccation and storage under vacuum, despite the absence of moisture, nutritive substances, and atmospheric oxygen. The viable properties of microbes in dry cultures may be preserved for an exceedingly long time. This is plainly confirmed by the fact that as the length of storage time of dry cultures increases the number of viable microbial cells decreases gradually. This may be explained by the fact that the processes of decay, in the dry cultures take place very slowly and that in essence they even contribute to lengthening the preservation period for the viable properties.

For instance, the principal and requisite conditions under which anabiosis in microorganisms is possible are a protective protein colloid, deep vacuum, and the drying factor.

From our experiments it can be seen that the selection of a drying medium such as the protective protein colloid is of great importance in preserving the viable properties of this or that dry culture. For instance, normal serum as a protective medium during desiccation was most effective, providing for a longer preservation of vital properties in the microbic cultures in the dry state. It must be indicated that we achieved positive results in all cases where we used normal serum as a protective medium for desiccation of some other microbial cultures.

Although gelatin has pronounced protective characteristics as a drying medium during freezing and drying, the dying of the microbes dried with gelatin takes place much faster than in cultures dried with serum. We also noticed manifestations of lysis of microbial cells which were confirmed by a macro or microscopic examination of the dissolved cultures and stained smears.

The lysis of cultures dried with gelatin and sucrose has also been indicated by other investigators. For example, Yu. A. Kozlov, B. S. Del'nik, and V. V. Vinogradov used gelatin and sucrose as a drying medium and determined the lysis of microbes when they were preserved in a dry state. This process was connected by the authors to the presence in the dry cultures of a marked percentage of residual moisture (3-7%).

Such a view of the lysis of microbial cultures dried with gelatin is apparently correct since the more moisture in dry cultures the more rapidly they die. As for the amount of residual moisture indicated by the authors (3-7%), it must be considered rather high, for our experiments in determining the amount of residual moisture in cultures dried with serum showed that the residual moisture did not exceed 0.4-0.7% to 1.0-1.5%. When cultures were dried with gelatin and sucrose the amount of residual moisture was likewise large (2.7%, 3.4%, 4.17%). We also noticed that gelatin as a protein colloid lends itself much less easily to drying.

The majority of bacterial cultures which we dried were facultative anaerobes. As our experiment showed, their preservation in a dry state without admission of free oxygen, that is, in a vacuum, not only did not damage them but on the contrary had a propitious effect on lengthening the preservation of vegetative, cultural biochemical, and virulent properties. For instance,

cultures dried with a serum colloid and kept under vacuum for 5-6 years at room temperatures, possessed good vegetative properties and a high virulence when tested. At the same time other samples of these same cultures but preserved in vacuumless ampules filled with dry air produced no growth when tested after 15 months.

The results of these experiments are produced in Table 3.

TABLE 3  
COMPARATIVE DATA ON TESTING DRY MICROBIAL CULTURES  
PRESERVED UNDER VACUUM AND WITHOUT VACUUM

Name of Culture	Strain No	Gas Medium	Length of Storage (in months)	Results of Seeding Nutritive Media
Salmonella enteritidis	5	vacuum	75	intense growth
		dry air	15	no growth
Salmonella cholerae-suis	5	vacuum	75	intense growth
		dry air	15	no growth
Poultry septicemia culture	115	vacuum	48	intense growth
		dry air	15	no growth
Sheep septicemia culture	4,519	vacuum	48	intense growth
		dry air	15	no growth
Streptococcus equi	D-9/1	vacuum	66	intense growth
		dry air	15	no growth

We see from the table that the resistance of the dry microbial cultures in the preservation of their viable properties depends on the presence or absence of atmospheric oxygen in the ampules. Where there was a vacuum in the ampules, the cultures produced intense growth when transferred to culture media, but where the ampules contained air dried by passing over newly regenerated calcium sulfate the cultures produced no growth. Other experiments showed that when dry cultures are kept in unsealed ampules with constant access of atmospheric oxygen, the dying process of the microorganisms is accelerated. Whereas microbes of dry cultures retained their viability for 15 months in ampules filled with dry air and sealed, those in unsealed ampules were good only for 6-7 months.

Accelerated drying of dry microbial cultures when stored without a vacuum has also been pointed out by other investigators. For instance, K. Ye. Dolinov, and L. B. Balayan found in testing the viability of dry pneumococcal cultures that microorganisms kept in dry air perished much earlier. For example, cultures kept under vacuum were viable for 38 months while those stored in ampules filled with dry air were viable for 6-7 months.

In this connection we must treat the problems of the decomposition of organic substances cited in the work of V. O. Tauson. In studying the decomposition (disintegration) of organic substances in peat bogs, V. O. Tauson came to the conclusion that the most rapid and complete decomposition takes place where there is considerable atmospheric oxygen available, that is, on the surface of the soil. In the deeper layers the decomposition of organic substances proceeds much more slowly since the availability of oxygen is limited by other organic substances, or rather decomposition products, lying in the upper layers.

For instance, experiments and observations by both us and other investigators have shown that one of the basic requirements for preserving microbial cultures in a dry state, in addition to dehydration and the use of a protective serum medium, is a deep vacuum during storage. The greater the access of atmospheric oxygen to the dry cultures, the more rapidly they die. The rapid destruction of dry microbial cultures when stored without vacuum may be explained by the fact that decomposition of protein in the cultures in this case takes place as the consequence of oxidation processes. When kept under deep vacuum the anabiotic state prevents the microbes from dying in large numbers, thanks to which their vital properties are preserved for a longer time.

In order to test the resistance of dry microorganisms to external factors we subjected them to low and high temperatures. For instance, dry cultures of paratyphoid fever of calves and pigs, hemorrhagic septicemia of sheep and hens, brucellosis of swine, and *Streptococcus equi* were placed in vacuum ampules in a refrigerator at a temperature of  $-17^{\circ}$ ,  $-20^{\circ}$  and kept in the cold for 28 months. After this time all these cultures possessed good vegetative properties when transferred to nutritive media. The results of this experiment showed that the prolonged action of low temperature does not have a harmful effect on the viability of dry microbial cultures.

Tests of the effect of high temperatures on dry microbial cultures were made at  $50^{\circ}$ ,  $75^{\circ}$  and  $105^{\circ}$ . In all cases the cultures were in sealed ampules under vacuum. The action of the  $50^{\circ}$  temperature continued unbroken for 40 days (Table 4).

TABLE 4

## EFFECT OF HIGH TEMPERATURE ON THE VIABILITY OF DRY CULTURES

Name of Culture	Strain No	Temperature of Thermostat	Length of Action in days	Results of Seeding on MPB (meat-pep- tone-broth) MPA (meat-pep- tone-agar)	
Septicemia of hens	1864	50	40	-	-
Septicemia of sheep	24	50	40	+	+
Septicemia of cattle	518	50	40	+	+
Brucellosis	39	50	40	+	+
Salmonella enteritidis	315/9	50	40	+	+
Salmonella cholerae-suis	9	50	40	-	-
Streptococcus equi	D-9/1	50	40	+	+
Streptococcus equi	D-9/2	50	40	+	+
Streptococcus equi	D-9/3	50	40	+	+

From Table 4 we can see that all the cultures with the exception of strains of hemorrhagic septicemia of hens and paratyphoid of pigs possessed vegetative properties after 40 days at a temperature of  $50^{\circ}$ .

At a temperature of 75° the cultures of Pasteurella remained viable for 3-5 hours and at 100-105° for 1-3 hours. Cultures of Streptococcus equi and paratyphoid fever of calves retained their viability for 5-8 hours at 75° and for 3-5 hours at 100-105°.

Thus the data from these experiments show that by providing new conditions for nonsporogenous microbial cultures, that is, by desiccation, one may obtain a new quality in them, a high resistance to high and low temperatures.

We must here point out that the cause of the very great resistance of dry microbial cultures is not only the fact that the microbes undergo total desiccation and are stored under vacuum but by the fact that they are dried in a protective protein colloid.

The use of the desiccation method for microbial cultures and their storage under vacuum radically change the medium in which they exist. The question arises as to whether such powerful factors as low temperature and almost total desiccation are able to affect the nature of the microorganisms by causing a change in their hereditary characters. If these characters of the microorganisms do not change is this not in contradiction with the Michurin doctrine that the effect of the environment is the principal factor which effects the hereditary characters of the organism.

Our experiments and the research of others in this field indicate that natural properties of microorganisms subjected to desiccation and prolonged storage under vacuum do not change. This can be explained by the fact that vital processes of dry microbial cultures kept under vacuum apparently come to a complete halt, where metabolism in the sense that we understand it is completely absent. But we know that the basis for the change in the

nature of a living organism is metabolism since the doctrine of T. D. Lysenko says "the cause of variation in the living body is a change in the type of acclimatization, in the type of metabolism. Change the type of metabolism of a living body and you will change its heredity pattern."

We have already pointed out that freezing and desiccation of microbial cultures under experimental conditions not only does not harm the vital properties of the microbes but on the other hand contributes to a preservation of their cultural biochemical, immunogenic, and virulent properties. For this reason questions of the desiccation and preservation of microbial cultures and living bacterial vaccines in the work of veterinary and medical microbiological institutions has not only practical but great theoretical importance. The significance of the theoretical answer to these questions lies not only in the fact that methods for drying microorganisms and living bacterial vaccines and their preservation in that state allows us to expand our knowledge of the life of microbes. Earlier concepts of the viability of nonsporogenous microorganisms following freezing desiccation were erroneous. The data from our experiments and those of other investigators offer evidence that nonsporogenous microorganisms may be kept for a long time in a frozen and dry state not only under experimental but under natural conditions.

As for experimental desiccation of microbial cultures, in this case the viability of nonsporogenous microbes has been preserved for years (5-6) by using a serum colloid protective medium during desiccation and by storing them under vacuum. It must be pointed out that microbial cultures in a dry state not only possess the capacity to grow when transferred to a culture medium but

preserve their cultural biochemical, virulent, and immunogenic properties. This confirms our hypothesis of anabiosis in minute living creatures microorganisms as a property which permits us not only to preserve their viability but to define the limits of their life. A study of the condition of dried microorganisms which have been preserved under vacuum for a long time shows that the limits of life of microbes goes far beyond the limits heretofore established by science and practice.

In surveying problems connected with anabiosis in microorganisms during desiccation we must deal briefly with the causes of the resistance of microbes. We must assume that the resistance of bacteria is not a chance phenomenon, that during the evolutionary process not only sporogenous but nonsporogenous microbes acquired the capacity of resisting desiccation under natural conditions. This is confirmed by the teachings of T. D. Lysenko on the effect of the environment on the formation of organisms and the development of hereditary characters in them. When subjected to desiccation under natural conditions, microbes like other organisms developed capacities for retaining viability without manifesting any visible life processes with which such phenomena as movement, propagation and, most important, metabolism are usually associated.

The organism greatly reduces its vital functions during desiccation since dehydration at first diminishes and then completely terminates processes of assimilation. Thus desiccation causes manifestations of great inhibition and possible cessation of life in the organisms, as believed by some investigators, and this is classified as anabiosis.

From what we said at the beginning of this chapter we saw that the more complex and highly organized the organism the more

rapid irreversible changes take place during desiccation. This means that manifestations of life and death in microbes and other organisms must be associated with the fact that their viability is preserved in those instances where irreversible processes have not taken place. In colloid chemistry, for example, we distinguish between reversible and irreversible colloids. Following desiccation reversible colloids are totally soluble in water while irreversible colloids settle down in the form of a solid precipitate. As is the case with the desiccation of microbial cultures their viability apparently depends on reversible and irreversible changes. In the experimental desiccation of microorganisms reversible changes are easily produced as a consequence of the almost total removal of moisture, the use of a protective protein colloid, and the presence of a deep vacuum during storage. However we must not assume that reversible changes which take place in microbial cells during experimental desiccation make it possible to preserve their viability for an indefinite time. In this case the dying processes in the dry microbial cultures, that is, the development of irreversible changes, is gradually prolonged. This indicates that microorganisms in a dry state even at the limit of life are still viable when transferred to other conditions, that is, to a culture medium where their vital functions are completely restored.

In reviewing anabiosis from the biological point of view we must regard it as a physiological phenomenon, aimed at adapting the organism to unfavorable external conditions and acquired during the long course of evolution along with a number of other properties necessary for the life of the organism. In defining the condition of microorganisms during experimental desiccation as anabiosis, we must assume that microbial cells are at the very edge of life. This

condition in dry microorganisms is indicated by the absence of conditions under which those life processes usually take place which are associated with movement, propagation, and metabolism. In fact when microbial cultures are desiccated from a frozen state in a vacuum apparatus they are almost completely dehydrated and can be stored for years under vacuum, that is, in this case the basic conditions of life for anaerobic microorganisms are lacking: moisture, nutritive substances, and atmospheric oxygen.

In this case we may cite the well known statement of F. Engels: "Life is a way of existence of protein bodies, the essential factor of which is the constant exchange of substances with the external environment." (F. Engels, Dialektika prirody [Dialectic of Nature], 1950, Gospolitizdat, page 244).

However, we must not consider the microbial cells of dry cultures to be lifeless (dead) since when other conditions are created, that is, moisture, nutritive medium, and oxygen are present, their lives, from the point of view of development and other physical, chemical, and biochemical indications, proceed normally. This brings us closer to the concept of the essence of problems of life and death in microscopic creatures, the microbes, when in a dry state. The death of microorganisms is evidently not a simple termination of life processes. It is determined by the destruction of vital elements in the organism with irreversible changes characterized by the decay and decomposition of protein substances.

As mentioned earlier, our experiments proved the great role of normal serum as a protective medium in the desiccation and preservation of bacterial cultures. The effectiveness of a serum

medium as a protein colloid may in this instance be explained by the fact that around the microbial cell we apparently create a membrane which protects the cell from the unfavorable action of external factors.

Thus in drying bacterial cultures and preserving them in the dry state a protective medium in the form of a protein colloid and deep vacuum contribute to a longer state of anabiosis in the microbial cells. In view of this fact, the concept of the viability of microorganisms which have been dried must be reviewed since bacterial cultures in a state of anabiosis and in a dry form possess an entirely new quality which is characterized by great resistance to external environmental factors. For this reason, when we look carefully at the problem of the life of minute organisms from the materialistic point of view, we must point out that the capacity for life is a feature of each protein body including those dry microbial cells which have not undergone decomposition.

In his work Anti-Duehring, Engels says that "wherever there is life, we find that it is associated with some protein body, and wherever there is a protein body not in the process of decomposition, we are, without exception, seeing manifestations of life" (F. Engels, Anti-Duehring, 1951, Gospolitizdat, page 77).

We believe that in the theoretical concept of the problems of anabiosis, that is, the viability of microorganisms in a dry state, we must start with the tenet of Engels since his treatment of life is unsurpassed in its theoretical and methodological significance. It is doubtless thoroughly applicable to both obvious and concealed forms of manifestation of viability in microorganisms, that is, to their anabiosis.

## CHAPTER V. DRY MICROBIAL CULTURES AND THEIR BIOLOGICAL PROPERTIES

Research on the production of dry microbial cultures and a study of their properties was first undertaken by us in 1944. Before this time experiments on drying certain microbial cultures had been carried out by Soviet investigators, in the field of medical microbiology. There is however no information on similar work in the realm of veterinary practice. However methods for keeping labile cultures as used in the practice of scientific institutes and biological preparation plants were in need of improvement. As we know, for research work in institutes and for the preparation of biological preparations in commercial plants we need to use microbial cultures possessing certain morphological, cultural, biochemical, antigenic, and virulent properties. This is one of the prerequisites provided for by all instructions and directions both for producing vaccine preparations and in hyperimmunization of producers of therapeutic and prophylactic, and diagnostic sera. In the meantime there are not infrequently cases in practice where these properties of microbial cultures undergo great modification during storage. In addition we must point out that in order to preserve certain microbial cultures as, for instance, pathogens of glanders, pasteurellosis, brucellosis and other labile microorganisms, frequent transfers to special culture media of a specific quality are required. This situation not only causes technical difficulties but likewise creates conditions favorable to the variation of microorganisms under the influence of the environment.

In solving these problems our biology starts, as we know, from the correct theoretical concept and practical observations of the life of living substances under natural conditions. In

developing new principles for preserving the cultural and biological properties of producing cultures we have selected as our basis the materialistic doctrine of I. V. Michurin and T. D. Lysenko on the effect of the environment on the vital capacities of microbial cells. For this reason we found it necessary to study the effect on the microbial cell of conditions which are created during desiccation and subsequent storage. That this influence must vary can be seen from the features of the problem and for this reason we have given very serious attention to this aspect.

In the chapter on anabiosis in microorganisms it has already been pointed out that our experiments established the effectiveness of a serum medium in drying and preserving cultures of the calf paratyphoid pathogen. In this case we believe it necessary to cite only some illustrative data on the degree of viability of calf paratyphoid microbes subjected to the action of low temperature and desiccation. As already indicated, for a protective medium we added to the culture in one case normal serum in a 1:1 ratio, in another a 6% solution of gelatin in the same proportions and in a third case a physiological solution. A count of the number of viable microbial cells following exposure to low temperatures for 2 hours and the termination of the drying process showed that many more microbes die during the freezing process than during desiccation as we see from the following data.

	Number of Viable Microbial Cells				
	Before freezing (in 1,000)	After freezing in 1,000	After desiccation in%	in 1,000	in%
Drying Medium					
Serum	86,800	52,080	60	41,664	48
Gelatin	93,140	51,300	55.1	41,300	44
Physiological solution	87,500	11,900	13.6	6,400	7.3

The dry culture of paratyphoid pathogen, kept under vacuum at room temperature, was later tested after 12, 24, and 36 months with the following results obtained.

Number of Living Microbial Cells

Drying Medium	Gas Medium	after 12 months		after 24 months		after 36 months	
		in 1,000	in%	in 1,000	in%	in 1,000	in%
Serum	vacuum	37,300	41.8	30,000	34.5	27,400	30.4
Gelatin	vacuum	38,100	38.7	12,180	13.0	no growth	-
Physiological solution	vacuum	4,100	4.5	3,200	3.7	no growth	-

From the data cited we can see that a culture dried with serum and kept for 3 years under vacuum contained 30.4% viable cells. A culture dried with gelatin and physiological solution produced no growth when tested after 3 years storage under the same conditions. For this reason we used as a basis in further research a drying method employing the addition of normal horse serum as a protective medium.

Before passing to an analysis of the data on the study of the cultural and biological properties of microorganisms subjected to desiccation we believe it necessary to treat briefly the general principles of the desiccation of microbial cultures which are employed in our experiments and in the work of the State Institute of Control over Veterinary Preparations.

#### Methods for Drying Microbial Cultures

We studied the effect on microbial cultures of the desiccation cycle, the protective protein media, residual moisture, and, for further preservation, vacuum. This gave us the opportunity to take into consideration the negative and positive aspects of the drying method in developing a new approach which would contribute to better preservation of microorganisms which we used in further work on drying many other microbial cultures.

Microbial cultures are cultivated for drying by the usual methods. In order to prepare a suspension of the microorganisms grown on solid culture media we made a physiological saline solution wash in a ratio of one ml of solution to one sq cm of surface of the culture. The cultures of microorganisms cultivated in a liquid culture medium were used in the form of a precipitate or a suspension.

Before drying we took cultures tested macro and microscopically for purity and uniformity of growth and added inactivated normal horse serum as a protective medium in a ratio of one part culture to one part serum or one part culture and 2 parts serum.

These proportions, as our studies showed, give full assurance of prolonged preservation of the biological properties of microorganisms dried in serum. Microbial suspensions under sterile conditions are poured into ampules at the rate of 0.3-0.5 ml per ampule, the ampules are joined by a vacuum tube to the connectors, pinched shut with pincers and frozen in a cooling mixture at a temperature of  $-20^{\circ}$  for 10-15 minutes. After freezing, the connectors with the cultures are attached to the manifold apparatus and the drying process begins. After 3-4 hours the first drying phase is usually considered as finished. The dishes with the cooling mixture are removed and the drying process continues at room temperature for 8-10 hours until total desiccation is achieved.

After 12-14 hours of drying microbial cultures usually contain a minimal amount of residual moisture (from 0.4-0.7% to 1.0-1.5%). The physicochemical properties of the cultures likewise have not undergone substantial changes (more of this later).

Over 4 years we repeatedly examined the vegetative, cultural, biochemical, agglutinative, and virulent properties of the dry

culture obtained with studies taking place at 3, 6, 9, 12, 18, 24, 30, 36, and 48 months after preparation. During the first 12 months of preservation the dry cultures were examined every 3 months. This was brought about by the necessity of checking the diverse data on the possibility of storing cultures in the dry state which had been found in the literature and, in addition, in order to get a more precise idea of the storage limits of the dry microbial cultures prepared. Our studies showed as early as the first year of storage that the dried strains could retain for a long time their original biological properties. For this reason the dry cultures were tested much less often afterwards.

In view of the fact that during 4 years the results of all experiments on the cultural, morphological, biochemical, agglutinative, and virulent properties of dry microbial cultures invariably showed the same indexes we will be satisfied with essentially a brief exposition of the data obtained during the final experiments of 1948. The results of testing the vegetative and virulent properties of certain dry cultures after 5-6 years of storage will be given in addition when the appropriate microorganism species is discussed.

#### Dry Cultures of the Calf Paratyphoid Fever Pathogen

For study we selected 5 production strains of the pathogen of calf paratyphoid fever, namely Nos 5, 7/1, 8/1, 10, and 315/9. Before desiccation the strains were tested for morphological, cultural, biochemical, agglutinative, and virulent properties. There was growth 24 hours after the cultures were transferred to meat-peptone-agar and meat-peptone-broth: on the agar in the form of round transparent S-shaped colonies and in broth in the form of intense turbidity of the culture with the formation of a loose bacterial precipitate on the bottom of the test tube.

Microorganisms of a 24-hour broth culture possessed pronounced mobility, had the appearance of short rods with rounded ends and did not take a stain by Gram's method. In media containing sugars the microorganisms split glucose, dulcitol, rhamnose, maltose, and mannite with the formation of acid and gas. They did not decompose lactose, sucrose, or raffinose, nor did they coagulate milk. When tested with a specific agglutinative serum the cultures had pronounced agglutinative properties.

The virulent properties of strains of the pathogen of calf paratyphoid fever were tested on white mice. The mice were inoculated subcutaneously with the wash from a 24-hour agar culture in doses of one and 10 million bacterial bodies according to the optical bacterial standard.

All 5 strains caused the death of all white mice inoculated subcutaneously in doses of one and 10 million bacterial bodies after 2, 3, 4, 5, 6, 7, and 8.5 days.

In all cases we found the original culture when the mice were dissected and the cultures incubated.

From the results of the biological tests it is evident that the cultures of the calf paratyphoid fever pathogen had virulent properties where white mice were concerned in doses as provided for by current regulations on the preparation and control of serum and vaccine for calf paratyphoid fever.

In order to have fresh cultures for desiccation, cultures of the calf paratyphoid pathogen tested for virulence were transferred to test tubes containing meat-peptone-agar and meat-peptone-broth. After macroscopic and microscopic examination for purity

and uniformity of growth the 24-hour culture was washed with a physiological saline solution. In order to produce a microbe suspension we added a protein colloid (normal horse serum) for a protective medium in the ratio of one part culture and 2 parts serum. After this the cultures were dried in a frozen state in a vacuum apparatus.

A test of the various properties of dried strains of the calf paratyphoid pathogen gave the following results.

Vegetative properties. Before transfer to culture media the ampules with dry cultures were tested for vacuum with a Tesla apparatus. In order to test vegetative properties the cultures, dissolved in a physiological saline solution, were transferred to test tubes with meat-peptone-agar and meat-peptone-broth. After 24 hours on the agar there were usually numerous S-shaped transparent colonies. In the meat-peptone-broth the culture grew intensely with the characteristic cloudiness of the medium and the formation of a loose bacterial sediment on the bottom of the test tube. As a result of the examinations it was determined that dry cultures possess well developed vegetative properties after 48 months' storage in a vacuum.

A microscopic examination of smears made with dry cultures following solution and smears made from 24-hour growths of these same cultures showed that the microorganisms of calf paratyphoid fever readily take a fuchsin stain but do not take a Gram stain. A study of 24-hour broth growths in a hanging or diluted drop showed that a large number of the bacterial cells possess pronounced mobility. Morphologically the bacteria of dry cultures of calf paratyphoid fever were short rods with rounded ends (Figures 24 and 25).

From the data cited from our experiments it is evident that after 4 years of storage the dry strains of the calf paratyphoid pathogen possessed typical vegetative, cultural, and morphological properties.

Biochemical and agglutinative properties. We thought it necessary to study in greater detail the biochemical and agglutinative properties of the dry cultures because these signs are the most typical of this microbe.

In order to test biochemical properties a 24-hour agar growth derived from dry strains was transferred to a medium containing sugars. The results of the transfer can usually be seen within 24 hours. However, in the case of some sugars, rhamnose, for example, the cultures were calculated after 2-3 days since some strains of the calf paratyphoid pathogen split this carbohydrate in a retarded fashion.

TABLE 5

## BIOCHEMICAL PROPERTIES OF DRY CULTURES OF THE CALF PARATYPHOID FEVER PATHOGEN

Strain No	Length of storage (months)	Name of Sugars								
		glucose	lactose	sucrose	dulcitate	rhamnose	maltose	raffinose	mannite	milk
5	48	A-G*	-	-	A-G	A-G	A-G	-	A-G	-
7/1	48	A-G	-	-	A-G	A-G	A-G	-	A-G	-
8/1	48	A-G	-	-	A-G	A-G	A-G	-	A-G	-
10	48	A-G	-	-	A-G	A-G	A-G	-	A-G	-
315/9	48	A-G	-	-	A-G	A-G	A-G	-	A-G	-

\*Formation of acid and gas

The data in Table 5 show that dry cultures of the pathogen of calf paratyphoid fever possessed typical biochemical properties after 4 years of storage.

The agglutinating quality of the dry cultures of calf paratyphoid pathogen were tested simultaneously with cultures kept on culture media by the usual method. To test tubes containing serum

of varying dilution we added the washings from a 24-hour agar culture obtained from dry strains until we had a concentration of 500 million bacterial bodies in one ml according to the optical bacterial standard.

The agglutination reaction was tested in one ml of liquid. Test tubes with this amount of liquid were placed in a thermostat for 6-8 hours after shaking. Then they were left at room temperature for 16-18 hours and afterwards the agglutination reaction was computed.

The results of testing the agglutinating quality of the dry cultures are shown in Table 6.

From the data cited in the table on a comparative test of the agglutinative properties of the pathogen of calf paratyphoid fever it is evident that cultures kept in a dry state possessed somewhat more pronounced agglutinative properties than cultures kept on meat-peptone-agar in the usual fashion. For instance, dry cultures 7/1, 5, and 8/1 showed a more pronounced reaction and in greater dilutions.

Virulent properties. The dry cultures were tested for virulence by inoculating white mice. In order to determine the virulence of the dry cultures of the calf paratyphoid pathogen white mice were inoculated with a 24-hour agar culture obtained from the dry strains. The mice were inoculated subcutaneously in doses of one and 10 million bacterial bodies according to the optical bacterial standard. The data from the test for virulent properties are presented in Table 7.

TABLE 6  
 AGGLUTINATIVE PROPERTIES OF DRY AND AGAR CULTURES OF THE PARATYPHOID PATHOGEN  
 AFTER 4 YEARS OF PRESERVATION

Strain No	Culture Preservation Method	Dilution of Agglutinating Serum						physiological solution
		1:100	1:1,000	1:2,000	1:4,000	1:6,000	1:8,000	
5	in a dry state	++++	++++	++++	++++	++++	+	-
5	transfer to agar (control)	++++	++++	++++	++++	+	-	-
7/1	in a dry state	++++	++++	++++	++++	+++	+++	-
7/1	transfers to agar (control)	++++	+++	+++	++	-	-	-
8/1	in a dry state	++++	++++	++++	+++	++	+	-
8/1	transfers to agar (control)	++++	+++	+++	+++	+	-	-
10	in a dry state	++++	++++	++++	++++	+	-	-
10	transfers to agar (control)	++++	++++	++++	++++	+++	-	-
315/9	in a dry state	++++	++++	++++	++++	+++	+	-
315/9	transfers to agar (control)	++++	++++	++++	++++	+++	+++	-

TABLE 7

## VIRULENT PROPERTIES OF DRY CULTURES OF THE PARATYPHOID PATHOGEN

Strain No	Length of pre-servation (months)	No of white mice	Dose	Died as Result of Inoculation
5	48	2	1 million	2 after 3.5-5.5 days
		2	10 million	2 after 2.5-4.5 days
7/1	48	2	1 million	2 after 4-8.5 days
		2	10 million	2 after 3-5.5 days
8/1	48	2	1 million	2 after 5.5-6.5 days
		2	10 million	2 after 3.5-6 days
10	48	2	1 million	2 after 5.5-6.5 days
		2	10 million	2 after 3.5-7.5 days
315/9	48	2	1 million	2 after 2.5-4.5 days
		2	10 million	2 after 2.5-3.5 days

As we see from the table strains of dry cultures caused death in all inoculated mice within 3-8 days. The pathogen of calf paratyphoid fever was recovered from the dead mice during bacteriological examination in all cases. The test showed that cultures of this pathogen after 4 years of storage in a dry form possessed the same virulent properties as before desiccation. The data presented in Table 7 with relation to virulent properties are confirmed by analogous experiments made during the entire preservation period. For instance, when tested at various times during storage (3, 6, 12, 24, 30, 36, and 48 months) the cultures of calf paratyphoid fever in a dry state invariably showed very pronounced virulence, causing death in white mice in the time established by regulations.

The results of these experiments are presented in Table 8.

TABLE 8

COMPOSITE DATA ON A TEST OF THE VIRULENT PROPERTIES OF DRY CULTURES  
OF THE CALF PARATYPHOID PATHOGEN AFTER 4 YEARS OF PRESERVATION

Strain No	No of experiments	No of white mice	Dose	Died as Result of Inoculation
5	7	14	1 million	14 after 3.5-6 days
	7	14	10 million	14 after 2.5-5 days
7/1	7	14	1 million	14 after 4.5-8.5 days
	7	14	10 million	14 after 3.0-6.5 days
8/1	7	14	1 million	14 after 5.5-6.5 days
	7	14	10 million	14 after 3.5-6.5 days
10	7	14	1 million	14 after 5.0-6.5 days
	7	14	10 million	14 after 3.5-7.5 days
315/9	7	14	1 million	14 after 2.5-6 days
	7	14	10 million	14 after 2.5-5.5 days

An additional test of the dry cultures of the calf paratyphoid pathogen made at the end of 1950, that is, 5.5 and 6 years after desiccation, showed that 4 of the 5 strains possessed good vegetative properties and caused death in white mice when given in the established doses. The fifth strain possessed good vegetative properties but was avirulent.

Dry Cultures of the Pig Paratyphoid Fever Pathogen

For purposes of study we used 5 productive strains of the pathogen of paratyphoid fever in pigs, Nos 3p, 5p, 7p, 9, and 10. Before desiccation the strains were tested for cultural, biochemical, agglutinative, and virulent properties.

Twenty-four hours after transplantation to meat-peptone-agar and meat-peptone-broth culture growth could be seen, on agar in the form of transparent S-shaped colonies and on broth in the form of intensive cloudiness of the medium.

The microorganisms of a 24-hour broth culture possessed pronounced mobility. In smears stained with fuchsin the microbes showed up as short rods with rounded ends. The pathogen of pig paratyphoid did not take a Gram stain. The cultures split glucose, rhamnose, maltose, and mannite with the formation of acid and gas. They did not decompose lactose, sucrose, dulcitol, or raffinose, did not coagulate milk, but possessed good agglutinative properties.

Before desiccation the virulence of the pig paratyphoid strains was tested on white mice. The mice were inoculated subcutaneously with a wash from a 24-hour agar culture in doses of one and 10 million bacterial bodies according to the optical bacterial standard. All 5 strains caused the death of all white mice in the doses employed between 3, 5, 6, and 8 days.

The cultures of the paratyphoid pathogen which underwent desiccation were grown in a thermostat on meat-peptone-agar and, for control, on meat-peptone-broth. After macro and microscopic testing for purity and uniformity of growth the agar growths were washed with a physiological saline solution. To the suspension of bacteria obtained we added normal horse serum as a protective medium in the proportions of one part bacterial suspension and 2 parts serum. After this the cultures were placed in ampules and freeze-dried in a vacuum apparatus.

Further examination of the dried strains of the pig paratyphoid culture produced the following results.

Vegetative properties. After testing the ampules with the culture for vacuum and dissolving the contents in a physiological saline solution the cultures were examined for vegetative properties

by transferring to culture media. Twenty-four hours after transfer to agar and broth the cultures were growing: on meat-peptone-agar in the form of numerous round transparent S-shaped colonies and on meat-peptone-broth with the characteristic cloudiness of the medium. As a result of these experiments we established that the dry cultures possessed pronounced vegetative properties when tested after 48 months of storage under vacuum.

Microscopy of smears prepared from dry cultures after solution in a physiological solution and those obtained from 24-hour growths on agar and broth established that the microorganisms which cause pig paratyphoid fever take a good fuchsin stain but do not take a Gram stain. An examination of 24-hour broth growths in hanging drops or diluted drops revealed a large number of microbial cells possessing pronounced mobility. Morphologically the bacteria of paratyphoid fever of pigs were short rods with rounded ends.

Thus we see that after 4 years of storage the dry cultures of the pig paratyphoid pathogen possessed typical vegetative, cultural, and morphological properties.

Biochemical and agglutinative properties. Of special interest is a study of the biochemical and agglutinative properties of dry cultures of pig paratyphoid fever since these signs are most typical and constant in identifying this species of microbe. The biochemical properties of dry cultures were tested by transferring 24-hour agar growths obtained from dry strains to culture media containing sugars.

TABLE 9

## BIOCHEMICAL PROPERTIES OF DRY CULTURES OF THE TIG PARATYPHOID PATHOGEN

Strain No	Length of storage (months)	glucose	lactose	sucrose	dulcitate	Name of the Sugar					milk
						rhannose	maltose	raffinose	mannite		
3p	48	AG*	-	-	-	AG	AG	-	AG	-	
5	48	AG	-	-	-	AG	AG	-	AG	-	
7p	48	AG	-	-	-	AG	AG	-	AG	-	
9	48	AG	-	-	-	AG	AG	-	AG	-	
10	48	AG	-	-	-	AG	AG	-	AG	-	

\*Acid and gas produced

TABLE 10

## AGGLUTININATIVE PROPERTIES OF DRY AND AGAR CULTURES OF THE TIG PARATYPHOID PATHOGEN AFTER 4 YEARS OF PRESERVATION

Strain No	Method of Preservation	1:100	1:1,000	1:2,000	Dilution of Agglutinative Serum				PS*
					1:4,000	1:6,000	1:8,000	1:12,000	
3p	in a dry form	++++	++++	++++	++++	++++	++++	++++	-
3p	by transfers to agar (control)	++++	-	-	-	-	-	-	-
5	in a dry form	++++	++++	++++	++++	++++	++++	+++	-
5	by transfers to agar (control)	+++	++	++	+++	+++	+	-	-
7p	in a dry form	++++	++++	++++	++++	++++	++++	++++	-
7p	by transfers on agar (control)	+++	+++	+++	+++	+++	+++	+++	-
9	in a dry form	++++	++++	++++	++++	++++	++++	++++	-
9	by transfers on agar (control)	++++	++++	++++	++++	++++	++++	++++	-
10	in a dry form	++++	++++	++++	++++	++++	++++	+++	-
10	by transfers on agar (control)	++++	++++	++++	++++	++++	++++	++++	-

\*PS: physiological solution

The data shown in Table 9 indicate that all the strains of dry cultures of the pig paratyphoid pathogen which we examined possess biochemical properties typical of this pathogen.

We tested the dry cultures for agglutinative properties by using the well known active specific agglutinative serum. For a control we tested cultures preserved by the usual method on nutritive media.

To a test tube containing different dilutions of agglutinative serum we added a wash from a 24-hour agar culture, obtained from dry strains, until we had a concentration of 500 million bacterial bodies in one ml according to the optical bacterial standard. The agglutination test was made in a one ml volume. The test tubes with this amount of liquid were shaken and placed for 6-8 hours in a thermostat and then kept at room temperatures for 16-18 hours until the reaction was read. The results of this experiment are presented in Table 10.

The results of a comparative study established that dry cultures of the pig paratyphoid pathogen retained pronounced agglutinative properties during 4 years of preservation. From Table 10 we see that strain No 3p of the pig paratyphoid pathogen completely lost its agglutinative properties during the storage period. The loss of agglutinability by strains kept under ordinary conditions on nutritive agar is not an exception since the loss of some biological properties by cultures is inevitable when they are preserved by transfer from medium to medium. The loss of agglutinative properties by strain No 3p when kept on a solid nutritive medium and the retention of these properties by the dry strains confirms the tenet that the desiccation of bacterial cultures permits the preservation of biological properties for a very long time.

Virulent properties. In order to test the virulent properties of dry cultures of the pig paratyphoid pathogen we used a 24-hour growth on agar to inoculate white mice. The culture was injected subcutaneously in doses of one and 10 million bacterial bodies. The results of testing the virulent properties of dry cultures are cited in Table 11.

TABLE 11

## VIRULENT PROPERTIES OF DRY CULTURES OF THE PIG PARATYPHOID PATHOGEN

Strain No	Length of storage (months)	No of white mice	Dose	Dead as a Result of Inoculation
3p	48	2	1 million	2 after 5.5-7.5 days
		2	10 million	2 after 4.5-5.5 days
5	48	2	1 million	2 after 4.5-5.5 days
		2	10 million	2 after 4.5-6.5 days
7p	48	2	1 million	2 after 2.5-5.5 days
		2	10 million	2 after 5.5-6.5 days
9	48	2	1 million	2 after 6.5-7.5 days
		2	10 million	2 after 2.5-3.5 days
10	48	2	1 million	2 after 4.5-5.5 days
		2	10 million	2 after 4.5-6.5 days

Cultures of the pig paratyphoid pathogen caused the death of all inoculated mice within 2.5-7.5 days, with the original culture recovered by bacteriological examination in all cases. Thus the experiments showed that the dry cultures retained a high virulence for 4 years.

It should be pointed out that dry cultures of the pig paratyphoid pathogen were tested for virulence over a period of 4 years at 3, 6, 12, 24, 30, 36, and 48 months.

The results of the experiments are provided in composite Table 12.

TABLE 12

COMPOSITE DATA ON TESTING THE VIRULENT PROPERTIES OF DRY CULTURES  
OF THE PIG PARATYPHOID PATHOGEN DURING 4 YEARS OF PRESERVATION

Strain No	Number of experiments	No of white mice	Dose	Died as the Result of Inoculation
3p	7	14	1 million	14 after 5.5-7.5 days
	7	14	10 million	14 after 4.5-6 days
5	7	14	1 million	14 after 4.5-5.5 days
	7	14	10 million	14 after 4.5-7 days
7p	7	14	1 million	14 after 2.5-7 days
	7	14	10 million	14 after 5.5-8 days
9	7	14	1 million	14 after 6.5-8.5 days
	7	14	10 million	14 after 2.5-5.5 days
10	7	14	1 million	14 after 4.5-5.5 days
	7	14	10 million	14 after 4.5-7.5 days

Repeated testing of dry cultures by inoculation of white mice invariably showed the high virulence of the strains studied. The mice died in all cases during the same periods of time.

This fact in turn confirms the pronounced constancy of the virulent properties of dry cultures of the pig paratyphoid pathogen when kept for 4 years.

Dry Cultures of the Hemorrhagic Septicemia Pathogen

In order to make the study we chose 8 productive strains including 2 strains of hemorrhagic septicemia of hens, 2 strains of hemorrhagic septicemia of sheep, 2 strains of hemorrhagic septicemia of swine, and 2 strains of hemorrhagic septicemia of cattle. Before desiccation the strains were tested for cultural, morphological, biochemical, and virulent properties with all the Pasteurella strains corresponding to established requirements. For instance, on meat-peptone-broth the cultures grew in the form of a uniform murkiness with subsequent formation of a slimy precipitate on the bottom of the test tube which rose in the form of

braids when the tubes were shaken. After 24 hours on meat-peptone-agar the cultures grew in the form of small, round, dew-drop colonies which later fused together forming a thin grey-white deposit. Under the microscope in the stained smears of the culture the field of vision showed polymorphic bacteria in monodiplococcal and monodiplobacillary forms.

In biochemical properties the cultures of *Pasteurella* corresponded to established requirements, that is, they split glucose and sucrose with the formation of acid. They did not decompose lactose, maltose, or dulcitol and did not coagulate milk.

The virulence of *Pasteurella* was tested on white mice through subcutaneous inoculation with a 24-hour broth culture in a 0.2 ml dose of 1:1,000 dilution.

As a result of the study we established that the cultures used for the experiment possessed virulence, that is, they caused the death of white mice in a positive period of time. For instance, strains Nos 115 and 452 of hemorrhagic septicemia of hens caused the death of all mice within 18, 20, 22, and 24 hours after inoculation. Strains Nos 24 and 4,519 of hemorrhagic septicemia of sheep caused death within 24, 38, 48, and 60 hours. Strains Nos 25 and 252 of hemorrhagic septicemia of swine caused death within 36, 40, 48, and 60 hours and strains Nos 518 and 858 of hemorrhagic septicemia of cattle caused the death of all mice after 40, 44, 48, and 80 hours. In all cases the culture used for inoculation was recovered from the dead mice.

For desiccation the cultures of *Pasteurella* were grown on meat-peptone-broth for 24 hours and, for purposes of control, on meat-peptone-agar. After macroscopic and microscopic testing for

purity and typicalness of growth, normal serum was added to the broth as a protective medium in the proportions which we always used. The cultures thus prepared were freeze-dried in a vacuum apparatus after packing in ampules. By examining the dry Pasteurella cultures 4 years after desiccation we obtained the following results.

**Vegetative properties.** After testing the ampules with cultures for vacuum and dissolving the contents in a physiological saline solution the cultures were examined for vegetative properties by seeding meat-peptone-agar and meat-peptone-broth. After 24 hours both the meat-peptone-agar and meat-peptone-broth, as a rule, showed pronounced growth. In the meat-peptone-broth the cultures grew in the form of uniform mild cloudiness with the formation of a slimy sediment on the bottom of the test tube. On the meat-peptone-agar the culture grew in the form of tiny round dew-drop colonies in an S-shape. In cases of overall growth of the culture on agar there was a delicate greyish-white bloom.

A microscopic examination of smears made from dry cultures following solution and smears of 24-hour growths established that the microorganisms take a good Giemsa and Muromtsev stain but do not stain by Gram's method. The microbes were monodiplococcal and monodiplobacillary shapes. There was no mobility of the microbes in a hanging drop preparation.

From the data cited we see that cultures of Pasteurella kept in a dry state for 4 years possessed typical vegetative, cultural, and morphological characteristics.

**Biochemical properties.** Although biochemical properties are not a very constant indication of typicalness in Pasteurella,

we still felt it practical to test cultures which had been kept in a dry state. With this purpose we made 24-hour broth cultures of Pasteurella obtained from dry strains in media with sugars, made with meat-peptone water or Hottinger's broth.

As a result of these experiments we determined that in biochemical properties the strains of Pasteurella which have been kept in a dry state for 4 years are typical. On the second and third days the cultures split glucose and sucrose with the formation of acid. They did not decompose lactose, maltose, or dulcitate and did not coagulate milk.

It must be mentioned that when Pasteurella is transferred to media containing sugar which have been made with peptone water there is a retarded capacity for splitting glucose and sucrose which was not observed where Hottinger's broth was used for the culture medium. This feature must apparently be explained by the intensive growth of Pasteurella in sugars prepared on Hottinger's broth and by the fact that the factor of accelerated acid formation was apparently related to the intensive propagation of the microbes.

Virulent properties. The virulence of Pasteurella cultures was tested by inoculation of white mice. For inoculation we used a 24-hour broth culture obtained from growths of dry strains. The mice were inoculated subcutaneously with a 0.2-ml dose of a 1:1,000 dilution. The results of these experiments are presented in Table 13.

From the table we see that Pasteurella strains which had been kept dry under vacuum for 4 years caused the death of all white mice in almost the same time as 4 years before, preceding desiccation.

TABLE 13

## VIRULENT PROPERTIES OF DRY PASTEURILLA CULTURES

Name of Culture	Strain No	Time of Storage (months)	No of mice	Dose of 1:1,000 dilution	Died as a Result of Inoculation
Pasteurella avium	115	48	3	0.2 ml	3 after 18-22 hours
	452	48	3	0.2 ml	3 after 16-24 hours
Pasteurella ovis	24	48	3	0.2 ml	3 after 20-28 hours
	4,519	48	3	0.2 ml	3 after 36-60 hours
Pasteurella suis	25	48	3	0.2 ml	3 after 36-36 hours
	252	48	3	0.2 ml	3 after 36-48 hours
Pasteurella bovis	518	48	3	0.2 ml	3 after 36-60 hours
	858	48	3	0.2 ml	3 after 36-48 hours

TABLE 14

## COMPOSITE DATA ON RESULTS OF TESTING THE VIRULENT PROPERTIES OF DRY PASTEURILLA CULTURES OVER A PERIOD OF 4 YEARS

Name of Culture	Strain No	No of Experiments	No of White Mice	Dose of culture (1:1,000)	Died as a Result of Inoculation
Pasteurella avium	115	7	21	0.2 ml	19 after 18-24 hours
	452	7	21	0.2 ml	21 after 16-26 hours
Pasteurella ovis	24	7	21	0.2 ml	21 after 20-30 hours
	4,519	7	21	0.2 ml	21 after 30-60 hours
Pasteurella suis	25	7	21	0.2 ml	21 after 36-48 hours
	252	7	21	0.2 ml	21 after 30-48 hours
Pasteurella bovis	518	7	21	0.2 ml	21 after 30-68 hours
	858	7	21	0.2 ml	21 after 28-60 hours
Total			168		168

During the 4 years of storage in a dry state under vacuum the virulent properties of the strains were tested 7 times (after 3, 6, 12, 24, 30, 26, and 48 months) and, as a rule, in all cases virulence was most pronounced.

The results of experiments are given in composite Table 14.

From the data in the table we can see that dry Pasteurella cultures showed high virulence when tested during the course of 4 years of storage. The cultures caused the death of the mice in minimal periods of time: from 16 to 24 hours for inoculation with hemorrhagic septicemia of hens and from 36 to 60 hours for inoculation with the other species of Pasteurella.

Available observations with regard to the inconstancy of the retention of virulence and other properties when microorganisms are kept by transfers to culture media served as a basis of comparison of virulent properties of dry microbial cultures with cultures kept by the usual method on culture media. According to our observations, after being kept for 3 years by numerous transfers on meat-peptone-agar, pig paratyphoid strain No 3p completely lost its agglutinative properties. Strain No 452 of a culture of hemorrhagic septicemia in hens showed greatly diminished virulence after preservation for 3 years by monthly transfers on meat-peptone-broth.

These facts regarding changes in the agglutinative and virulent properties of microorganisms when kept by the usual method are not isolated. Similar phenomena in microbiological practice have been constantly observed. However they have not been studied for the reason that it was difficult to make a comparative test of the biological properties of the same strains since there was no reliable method for preserving microbial cultures over a long period of time.

This hypothesis, as we shall see later, was confirmed by the very first experiments in making a comparative study of the virulent properties of Pasteurella cultures kept in a dry form and by transfers in meat-peptone-broth.

The causes for the decline in virulent properties in microbial cultures preserved by the usual method must be sought in the fact that culture media used for the transfers cannot be identical in physiochemical and other properties. In view of this fact, there is frequently sparse or atypical growth of cultures which inevitably causes changes in the biological properties including a diminution in virulence. In addition, there are indications in the literature that frequent transfers of microbial cultures on culture media lead in general to a change in their cultural and biological properties (Rechnenskiy, Klimentova, Fedorov).

We know from practice that even passages in animal organisms do not promote the preservation of virulent properties in certain microbial cultures kept by the usual method on nutritive media. This is confirmed by the fact that in order to preserve virulent properties productive cultures of microorganisms are passed yearly through animals and some of them, as the Pasteurella strains, 2-3 times a year but despite this their virulence very often declines markedly.

In order to make a comparative study of virulent properties we made several experiments with Pasteurella strains. Here we are indicating one of these experiments. For the experiment we used 3 strains kept in a dry state and 3 preserved by transfers in culture . broth.

The white mice were inoculated with a 24-hour broth culture in minimal and maximal doses.

The results of this experiment are produced in Table 15.

We see from the table that cultures of septicemia of hens, strains No 452 and 4,519, kept in a dry state for 38 months caused the death of white mice in a dilution of 1:100,000, 1:1,000,000, and 1:500,000 (for strain 4,519) in 16-36 hours while cultures of these same strains preserved in meat-peptone-broth by periodic transfers caused the death of white mice only in dilutions of 1:100, 1:1,000 and 1:10,000. Almost analogous results were obtained when the white mice were inoculated with a culture of septicemia of sheep (strain No 24) where the dry culture caused the death of white mice in a 1:100,000 dilution while a culture kept for the same period of time in meat-peptone-broth caused death in white mice in a dilution of 1:1,000.

In addition we must point out that cultures of septicemia of hens (strain No 452) and septicemia of sheep (strain No 4,519) which had been kept in culture broth were passed through the body of an appropriate animal species every year in order to increase virulence. Yet despite the passages their virulence dropped after 6 or 7 transfers.

In order to compare the virulent properties of dry microbial cultures of Pasteurella and cultures kept in meat-peptone-broth we are citing examples from work done at our institute and at the Stavropol' Biological Preparations Plant.

For the work of the laboratory on biological preparations for pasteurellosis and erysipelas and for distribution to biological preparations factories we prepared a number of productive strains

of Pasteurella at the beginning of 1947. At the laboratory mentioned these cultures were kept both in a dry form and on nutritive media. After 9 months the laboratory director, N. M. Nikiforova, a candidate in veterinary medicine, used in titrating a lethal dose a culture of hemorrhagic septicemia of cattle (strain No 796), kept in meat-peptone-broth, in order to make a control check of immunity in cattle previously vaccinated by her. This culture was but slightly virulent and did not cause the death of the animal. Then she used a dry culture of septicemia of cattle (strain No 796) for inoculating cattle for the same purpose. She found that the dry strain possessed great virulence. The same situation was found at the Stavropol'

Biological Preparations Plant where cultures of a number of strains of septicemia of hens kept in meat-peptone-broth showed decreased virulence in comparison with dry cultures.

The data in Table 15 thoroughly confirm our opinion that the best method for preserving Pasteurella cultures is desiccation. This method permits the preservation of cultural, biochemical and virulent properties for a very long time without having recourse to transfers and passages through the body of larger animals.

A subsequent test of the three strains of hemorrhagic septicemia of cattle (strains No 41, 518, and 858), one strain of hemorrhagic septicemia in hens (No 1,864), one strain of hemorrhagic septicemia in sheep (No 24) and one strain of hemorrhagic septicemia in swine (No 252) showed that these cultures possessed good vegetative properties, typical growth on nutritive media, and high virulence for white mice when examined 5 and 5.5 years after desiccation.

TABLE 15

## VIRULENT PROPERTIES OF PASTEURILLA CULTURES KEPT IN A DRY STATE OR IN MEAT-PEPTONE-BROTH

Name of Culture	Strain No	Storage (in months)	Preservation method	No of White Mice	Dose in ml.	Dilution of 24-hour Broth Culture	Died as a Result of Inoculation
Pasteurella avium	452	38	Dry under vacuum	2	0.2	1:100	2 in 8.5-15 hours
				2	0.2	1:1,000	2 in 24-24 hours
				2	0.2	1:10,000	2 in 20-36 hours
				2	0.2	1:100,000	2 in 28-29 hours
				2	0.2	1:1,000,000	2 in 16-36 hours
Pasteurella avium	452	38	Transfers on meat-peptone-broth	2	0.2	1:100	2 in 24-26 hours
				2	0.2	1:1,000	2 in 36-36 hours
				2	0.2	1:10,000	2 in 24-36 hours
				2	0.2	1:100,000	-
				2	0.2	1:1,000,000	-
Pasteurella avium	4,519	38	Dry under vacuum	2	0.2	1:100	2 in 36-36 hours
				2	0.2	1:1,000	2 in 36-36 hours
				2	0.2	1:10,000	2 in 36-36 hours
				2	0.2	1:100,000	2 in 36-48 hours
				2	0.2	1:500,000	2 in 36-36 hours
Pasteurella avium	4,519	38	Transfers on meat-peptone-broth	2	0.2	1:100	2 in 36-48 hours
				2	0.2	1:1,000	2 in 36-60 hours
				2	0.2	1:10,000	-
				2	0.2	1:50,000	-
				2	0.2	1:500,000	-
Pasteurella ovis	24	38	Dry under vacuum	2	0.2	1:100	2 in 16-24 hours
				2	0.2	1:1,000	2 in 24-36 hours
				2	0.2	1:10,000	2 in 24 hours
				2	0.2	1:100,000	2 in 24-36 hours
Pasteurella ovis	24	38	Transfers on meat-peptone-broth	2	0.2	1:100	2 in 36-36 hours
				2	0.2	1:1,000	2 in 48-72 hours
				2	0.2	1:10,000	2 in 36 hours
				2	0.2	1:100,000	-

### Dry Cultures of Brucella

~~For our study we selected 5 productive strains, 2 of the~~  
pathogen of brucellosis in cattle, 2 of the pathogen of brucel-  
losis in swine, and one of the pathogen of brucellosis in sheep.

Before desiccation the strains were tested for cultural,  
morphological, agglutinative, and virulent properties. On a  
meat-live sugar-glycerin agar culture the Brucella grew in the  
form of small convex greyish colonies. In broth, as a rule,  
there was a soft diffuse growth by the second or third day.

In smears made from cultures and stained by Kozlovskiy's  
method the Brucella had a bright red appearance with the back-  
ground a pinkish color. The Brucella microbes had a character-  
istic polymorphic quality, sometimes in the form of oval cocci,  
sometimes in the form of short rods with rounded ends.

The test for agglutinability showed that the Brucella  
strains selected for our experiment possessed pronounced agglu-  
tinative properties. For example, when Wright's reaction was  
made with active specific agglutinative serum the strains agglu-  
tinated with a ++ and +++ index in dilutions of 1:50 to 1:1,600.

In order to study the virulent properties of Brucella  
guinea pigs were inoculated with a 3-day agar culture in a 1-  
billion bacteria dose based on the optical bacterial standard.  
As a rule 7 or 8 days after inoculation the guinea pigs showed  
marked beginnings of illness with a drop in weight of from 20% to  
30%. Some 15-17 days after inoculation the guinea pigs were killed  
and examined bacteriologically and pathoanatomically. A Brucella  
culture was recovered from almost all the guinea pigs, from the  
spleen and somewhat less often from the cardiac blood. The ag-  
glutination reaction with blood serum from the killed guinea pigs  
showed a pronounced agglutination titer (++ and +++) in dilutions  
of 1:25, 1:50, 1:100, and 1:200.

For drying, the Brucella cultures were grown for 3 days on meat-liver sugar-glycerine agar. After macroscopic and microscopic tests for purity and typical growth, normal serum was added as a protective medium to a wash of the agar growth in the proportions of 1 part culture and 2 parts serum. After this the cultures were freeze-dried in a vacuum apparatus.

Further studies of the dried Brucella cultures produced the following results.

**Vegetative properties.** The contents of ampules tested for vacuum were dissolved in a physiological saline solution and transferred to a meat-liver sugar-glycerine agar culture. After 2 days, as a rule, the Brucella cultures had grown on the agar in the form of small transparent convex greyish S-shaped colonies. In meat-peptone-broth, growth in the form of a diffuse cloudiness of the broth ordinarily started on the second or third day.

Thus cultures of Brucella kept dry under vacuum for 4 years possessed most pronounced vegetative properties. It must be pointed out that following numerous transfers to nutritive media which we made for 4 years there was not a single case of attenuation in culture growth. This presents evidence of the fact that strains of dry cultures of Brucella possessed pronounced capacity for reproduction.

In smears made from broth and agar growths and stained by Kozlovskiy's method the microorganisms had a bright red color while the background was pinkish. The microbes had a characteristic polymorphic quality, sometimes in the form of oval cocci, sometimes in the form of short rods with rounded ends.

**Agglutinative properties.** We tested agglutinability in strains of dry Brucella cultures by Wright's reaction for which we

used the well known active specific agglutinating serum. A wash from a 3-day agar Brucella culture was added to test tube serum of various dilutions up to a concentration of 500 million bacterial bodies in one ml according to the optical bacterial standard. After shaking, the test tubes were placed in a thermostat for 18 hours and then kept at room temperature for 20 hours. As a rule, after 24 hours a large number of the test tubes showed a pronounced agglutination reaction. However in order to obtain more accurate results we made another reading of the reaction after 36-40 hours. The results of testing agglutinating properties of Brucella cultures are shown in Table 16.

From the table we can see that dry Brucella cultures kept for 4 years possessed pronounced agglutinative properties when they were tested with specific agglutinating serum.

Virulent properties. In order to test the virulent properties of dry cultures of Brucella we inoculated guinea pigs with a 3-day agar culture obtained from strains which had been preserved in a dry state. The cultures were injected subcutaneously in a dose of 1 billion bacterial bodies according to the optical bacterial standard. The results of these experiments are presented in Table 17.

A Brucella culture was recovered from the spleen and less often from the cardiac blood of almost all the guinea pigs. In the dead and killed guinea pigs there was marked enlargement of the lymphatic glands and in individual cases there was pronounced necrosis at the inoculation site.

TABLE 16

## AGGLUTININATIVE PROPERTIES OF DRY CULTURES OF BRUCELLA

Name of Culture	Strain No	Length of storage (months)	Dilution of Agglutinating Serum								Physiological solution
			1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	
Br. bovis	1,425	48	++++	++++	++++	++++	++++	++++	++++	++++	-
Br. bovis	4,004	48	+++	++++	++++	++++	++++	++++	++++	++++	-
Br. suis	1	48	+++	++++	++++	++++	++++	++++	++	++	-
Br. suis	39	48	+++	++++	++++	++++	++++	++++	++++	++	-
Br. melitensis	74	48	+++	++++	++++	++++	++++	++++	++++	+	-

TABLE 17

## VIRULENT PROPERTIES AND AGGLUTININABILITY OF DRY CULTURES OF BRUCELLA

Name of Culture	Strain No	Length of storage (months)	No of inoculated guinea pigs	Dilution of Blood Serum of Killed Guinea Pigs						Results of a Bacteriological Examination
				Died	Killed	1:25	1:50	1:100	1:200	
Br. bovis	1,425	48	2	-	2	++++	++++	++++	++++	Brucella culture recovered from 70-80% of guinea pigs
Br. bovis	4,004	48	2	2	-	++++	++++	++++	+++	
Br. suis	1	48	2	1	1	++++	++++	++++	++++	
Br. suis	39	48	2	-	2	++++	++++	++++	+++	
Br. melitensis	74	48	2	1	1	++++	++++	++++	+++	

The results of the agglutination reaction showed that blood serum from inoculated guinea pigs possessed a pronounced agglutination titer. Analogous examinations for virulence in dry Brucella cultures were made repeatedly during the 4-year period (after 3, 12, 15, 21, 24, 35, and 48 months). Data from these experiments are produced in composite Table 18.

The results of these experiments indicate that guinea pigs inoculated with an agar Brucella culture obtained from dry strains fell ill after 6-8 days with symptoms of exhaustion and a loss of weight by 20-30%. Dissection of dead and killed guinea pigs showed marked enlargement of the lymphatic glands and, in individual cases, even an enlarged spleen, and in 70-80% of the cases a Brucella culture was recovered. From the experiments analyzed in tables 17 and 18 we can see that dry cultures of Brucella possessed the capacity of stubbornly retaining their virulence for a very long time.

As a result of all our studies it was determined that cultures of the brucellosis pathogen kept for 4 years in a dry state possessed typical morphological, cultural, and agglutinative properties with no deviations from the normal in their virulence.

#### Dry Cultures of the Pathogen of Anaerobic Dysentery in Lambs

For our study we selected 2 strains of the pathogen of anaerobic dysentery in lambs (Nos 1 and 4). Before desiccation the strains were examined for cultural, morphological, virulent, and toxic properties.

In meat-liver broth under vaseline oil young cultures showed luxurious growth with gas formation 7-8 hours after seeding. Under the microscope smears and hanging drop preparations showed thick isolated rods taking a good Ziehl's fuchsin stain and a good Gram stain but lacking mobility.

TABLE 18

COMPOSITE DATA FROM TESTING THE VIRULENT PROPERTIES AND AGGLUTINABILITY OF DRY CULTURES

Name of Culture	Strain No	OF BRUCELLA DURING THE 4-YEAR STORAGE PERIOD		Dilution of Serum of Killed Guinea Pigs					Results of Bacteriological Examination
		No of inoculated guinea pigs	Died	Killed	1:25	1:50	1:100	1:200	
Br. bovis	1,425	12	1	11	++++	++++	+++	++	Brucella culture recovered from 70-80% of guinea pigs
Br. bovis	4,004	6		6	++++	++++	++++	++	
Br. suis	1	12	1	11	++++	++++	++++	++	
Br. suis	39	6		6	++++	++++	++++	+++	
Br. melitensis	74	12	2	10	++++	++++	++++	+++	
Total		48	4	44					

In testing the biochemical properties of original strains of anaerobic dysentery in lambs by seeding media containing sugar we determined that the cultures possessed the capacity to split glucose, lactose, sucrose, levulose, and maltose with the formation of acid and gas, did not change mannite and dulcitate and rapidly coagulated milk.

Guinea pigs inoculated subcutaneously with a 24-hour broth culture of the lamb dysentery pathogen in a 0.3 ml dose died after 2 days. At the injection site there developed characteristic pathoanatomical lesions in the form of pronounced edema in the region of the subcutaneous connective tissue. Rabbits inoculated intravenously with an 8-hour broth culture in a 0.2-ml dose died within 7-10 minutes from intoxication.

The tested cultures of the pathogen of anaerobic dysentery of lambs were transferred to meat-liver broth under vaseline oil and cultured for 12 hours. After macroscopic and microscopic examination for purity and typical growth normal horse serum was added to the culture in the proportion of one part culture and 2 parts serum. Microbial suspensions thus prepared were freeze-dried in a vacuum apparatus. Further examinations of the dried cultures produced the following results.

Vegetative properties. Ampules with a culture of the pathogen of lamb dysentery were tested for vacuum before transfer to culture media. After this the contents of the ampules were dissolved in a physiological saline solution and transferred to meat-liver broth under vaseline oil. After 6 to 8 and rarely 12 hours in a thermostat the cultures showed rampant growth with the formation of gas. Tests of lamb dysentery cultures kept in a dry state for 35 months showed that they possessed pronounced vegetative properties.

Cultural biochemical properties. A microscopic examination of cultures of lamb dysentery obtained directly from ampules after dissolving the contents and from transfer culture growths showed that the microorganisms are isolated thick bacilli (Figures 26 and 27) not possessing mobility. The microbes took good Ziehl fuchsin and Gram stains.

The dry cultures of lamb dysentery transferred to media containing sugars split glucose, lactose, sucrose, levulose, and maltose with the formation of acid and gas, did not change mannite and dulcitate, but rapidly coagulated milk.

Virulent and toxic properties. The virulent properties of dry cultures of lamb dysentery were tested by the inoculation of guinea pigs. The guinea pigs which were inoculated subcutaneously with a 0.2-ml dose of a 24-hour broth culture obtained from dry cultures died within 2-2.5 days and dissection showed a pathological picture characteristic of changes caused by these pathogens.

The toxicity of dry cultures of lamb dysentery was tested on rabbits. The rabbits were inoculated intravenously with 8 to 10-hour growths obtained from dry strains. As a result of the experiment we found that the cultures possessed very great toxicity. For instance, in a dose of 0.1, 0.2, or 0.3 ml the cultures caused death in rabbits from intoxication in 5, 6, 8, and 10 minutes. Seeding media with blood from the hearts of dead rabbits usually produced growth of a pure culture of the lamb dysentery pathogen.

Along with a study of the toxic properties of a fresh broth culture of the lamb dysentery pathogen we thought it very important to test the retention of toxin in the dry culture itself. In our opinion, this would enable us to establish the preservation not

only of vegetative and virulent properties in the dry state but the preservation of the toxin as well. For this purpose we injected rabbits intravenously with 0.2 ml of a dry culture dissolved in a physiological solution.

The results of testing the virulent and toxic properties of cultures of lamb dysentery are presented in Table 19.

From the table we see that dry cultures of anaerobic dysentery of lambs retained not only virulent but toxic properties for 35 months. Guinea pigs inoculated with a broth growth obtained from dry cultures died in the prescribed time. As a result of injecting a culture into the subcutaneous connective tissue of guinea pigs, edema developed as a rule.

In studying the toxic properties of dry cultures following 35 months of storage under vacuum it was established that broth growths obtained from dry cultures as well as the dry cultures themselves contain a sufficient amount of toxin to cause the rapid death of a rabbit following intravenous injection of a 0.2-ml dose. The preservation of toxic properties of cultures of the pathogen of lamb dysentery has been confirmed by numerous experiments during the course of 35 months (after 6, 12, 24, 30, and 35 months). The results of these experiments are presented in Table 20.

All our experiments brought out evidence of the possibility of preserving the cultural, morphological, virulent, and toxic properties of dry strains of the lamb dysentery pathogen for a long time.

TABLE 19

## VIRULENT AND TOXIC PROPERTIES OF DRY CULTURES OF THE PATHOGEN OF LAMB DYSENTERY

Name of Culture	Strain No	Length of Storage (months)	No of inoculated animals	Method of inoculation	Dose (ml)	Dead as a Result of Inoculation
24-hour broth culture*	1	35	2 guinea pigs	Subcutaneous	0.3	2 after 2-2.5 days
8-hour broth culture*	1	35	2 rabbits	Intravenous	0.2	2 after 6-8 minutes
Dry culture	1	35	2 rabbits	Intravenous	0.2	2 after 12-17 minutes
24-hour broth culture*	4	35	2 guinea pigs	Subcutaneous	0.3	2 after 2-2.5 days
8-hour broth culture*	4	35	2 rabbits	Intravenous	0.2	2 after 5-8 minutes
Dry culture	4	35	2 rabbits	Intravenous	0.2	2 after 8-12 minutes

\*Broth culture obtained by transferring a dry dysentery culture to meat-liver broth under vaseline oil.

TABLE 20

COMPOSITE DATA FROM TESTING TOXIC PROPERTIES OF DRY CULTURES OF LAMB DYSENTERY PATHOGENS  
DURING A 35-MONTH STORAGE PERIOD

Strain No	No of Experiments	No of Rabbits Inoculated	Inoculated with What	Inoculation Method	Dose (ml)	Died as a Result of Inoculation
1	5	8	6-8-hour broth culture*	Intravenous	0.1	8 after 5-8-10 minutes
4	5	8	6-8-hour broth culture*	Intravenous	0.2	
					0.3	8 after 5-8-9 minutes
1	3	6	dry culture	Intravenous	0.3	
					0.2	6 after 7-14-17 minutes
4	3	6	dry culture	Intravenous	0.3	
					0.2	6 after 6-8-12 minutes
Total		28			0.3	28

\*The broth culture was obtained by transferring a dry lamb dysentery culture to meat-peptone-broth under vaseline oil.

The capacity for preserving toxin in dry cultures of the lamb dysentery pathogen for almost 3 years is of very great significance for veterinary medicine since it permits us to use dry standard toxin for titering vaccine and serum for anaerobic dysentery of lambs. In addition, the drying method enables us to preserve the virulent and toxic properties of this pathogen for a long time in order to make high grade biological preparations.

A supplementary test of 2 strains of the pathogen of anaerobic dysentery of lambs (Nos 1 and 4) showed that the culture possessed vegetative and toxic properties when examined 5.5 years after desiccation. For instance, following intravenous inoculation with a 0.1-ml dose of dry lamb dysentery culture white mice died in 3-7 minutes. Rabbits inoculated intravenously with a 10-hour broth culture in a 0.3-0.5-ml dose died in 8-10-15 minutes from intoxication.

#### Dry Cultures of the Strangles Pathogen

As we know, preserving cultures of the strangles streptococcus in culture media is quite difficult. In practice it not infrequently turns out that the streptococci soon die when kept in culture media. This situation long ago brought out the necessity for having an improved method for keeping these cultures.

For our study we selected 3 strains of the strangles streptococcus (Nos D9/1, D9/2, and D9/3). Before desiccation the strains were tested for cultural, morphological and virulent properties.

Ordinarily for growing cultures of the strangles streptococcus we use meat-peptone-broth with normal horse serum added. However, we have recently started to use Muromtsev's semiliquid agar and this medium has been acceptable since the streptococci grow intensively in it.

In testing the cultural and morphological properties of strangles streptococcal strains grown on semiliquid agar and selected for the experiment we determined that a 24-hour growth consists of individual cocci and short chains.

The streptococcal strains caused the death of white mice 20-30 hours after intraperitoneal injection of a 0.1-ml dose.

For desiccation the tested streptococcal cultures were transferred to semiliquid agar where they were cultured for 24 hours. After macroscopic and microscopic tests for purity and typical growth pattern the cultures were split up into ampules and desiccated.

During our first experiment the streptococcal cultures were dried without any normal serum being added since we assumed that the semiliquid agar in an amount of 0.2% in which the streptococci were grown might serve as a protective medium during freeze-drying. However during storage it was determined that streptococcal cultures dried without the addition of normal serum did not possess the capacity for retaining their vegetative properties for a long time. For instance, two of the three dried strains did not grow when semiliquid agar was seeded with them after 12-15 months of storage.

Thus, the attempt to produce high grade dry cultures of streptococcus without the addition of normal serum for a protective colloid was unsuccessful in our experiment. Because of these circumstances the experiment in drying these strains of streptococcal culture was repeated but this time we used normal serum as a protective medium.

For purposes of desiccation the streptococcal cultures were cultivated in semi liquid agar for 24 hours. After a macroscopic and microscopic examination for purity and typical growth we added normal horse serum to the culture in the proportion of one part culture and 2 parts serum. These cultures were dried from a frozen state in a vacuum apparatus. Further examinations of the dry cultures of the strangles streptococcus produced the following results.

**Vegetative properties.** Before transferring to nutritive media the ampules with dry streptococcal cultures were tested for vacuum after which the cultures were dissolved in a physiological saline solution and transferred to semiliquid agar. Some 24 hours later there was usually luxuriant growth of the streptococcal culture on the agar. These experiments showed that cultures of strangles streptococci kept in a dry state for 3 years under vacuum possessed pronounced vegetative properties.

**Cultural and morphological properties.** Experiments aimed at studying cultural and morphological properties showed that 24-hour growths of streptococci obtained from dry strains contained isolated cocci and short chains. The streptococci took a good aqueous fuchsin and Löffler's blue stain. Surface streaked cultures of streptococcus on blood agar in Petri dishes showed pronounced growth after 24 hours in the form of tiny whitish colonies with a clear zone around them (hemolysis). This zone later increases markedly, reaching total hemolysis of all the erythrocytes in the agar toward the fourth or fifth day.

Dry cultures of streptococci, as shown by the experiments, possessed their characteristic weak biological properties. For instance, the streptococci split glucose and sucrose with the formation of acid, did not change lactose, maltose, raffinose, and mannite and did not coagulate milk.

Virulent properties. The virulent properties of dry cultures of streptococci were tested by inoculating white mice intraperitoneally with a 0.1-ml dose of a 24-hour growth cultured on semiliquid agar by transference of dry strains. The streptococcal cultures kept under vacuum in a dry state for 3 years possessed pronounced virulence, causing death in white mice within 20-26 hours. In all cases we were able to recover a culture of the strangles streptococcus from the cardiac blood of the dead mice.

The data from these experiments are presented in Table 21.

The virulent properties of dry streptococcal cultures were tested several times during the course of the 3-year storage period (after 3, 6, 14, 26, and 36 months). The results of these tests are shown in composite Table 22.

As we see from tables 21 and 22, the streptococcal cultures possess pronounced virulent properties. Thus it was established that desiccation allows the preservation for a long time of cultures of the strangles streptococcus, with the cultural, morphological, and virulent properties of the microorganisms undergoing no changes during the process.

An additional test of 3 strains of the strangles streptococcus (Nos D9/1, D9/2, and D9/3) 5.5 years after desiccation also showed that the dry cultures possess good vegetative properties and a high virulence for white mice.

TABLE 21

## VIRULENT PROPERTIES OF DRY CULTURES OF STREPTOCOCCI

Strain No	Length of Storage (months)	No of Mice Inoculated	Inoculation Method	Dose in ml	Died as a Result of Inoculation
D9/1	36	3	intraperitoneal	0.1	3 after 18-22-26 hours
D9/2	36	3	intraperitoneal	0.1	3 after 20-24-26 hours
D9/3	36	3	intraperitoneal	0.1	3 after 22-22-26 hours

TABLE 22

COMPOSITE DATA ON TESTING THE VIRULENT PROPERTIES OF DRY CULTURES  
OF STREPTOCOCCI DURING THE 3-YEAR STORAGE PERIOD

Strain No	No of Experiments	No of Mice Inoculated	Inoculation Method	Dose in ml	Died as a Result of Inoculation
D9/1	5	15	intraperitoneal	0.1	15 after 18-20-28 hours
D9/2	5	15	intraperitoneal	0.1	15 after 17-22-26 hours
D9/3	5	15	intraperitoneal	0.1	15 after 18-24-28 hours

As already indicated, for determining the virulent properties of dry microbial cultures we used growths on broth and agar obtained by cultivation of dry strains. However we thought it necessary to test the virulence of the dry strains directly. For this purpose we used dry cultures immediately after their dissolution in a physiological solution. As a result of our experiments we found that dry cultures kept for 32 months possessed virulent properties for white mice but in higher doses. For instance, while cultures of the pathogen of calf and pig paratyphoid fever obtained by cultivation from dry strains caused death in white mice in doses of one and 10 million bacterial bodies, the dry cultures administered directly caused death in white mice only in much larger doses. We found approximately the same virulent properties in the dry cultures of Pasteurella which caused death in white mice in a 0.2-ml dose of 1:100 dilution but did not cause death in a 1:1,000 dilution. This is explained by the fact that the number of living microbial cells in the cultures decreased markedly as a result of desiccation and prolonged storage.

#### Dry Cultures of the Malignant Anthrax Pathogen

Cultures of malignant anthrax are not labile but they are much more easily and simply preserved in a dry form. For this reason at the State Institute of Control all productive strains of malignant anthrax are kept and distributed to the biological preparations plants only in a dry form.

The malignant anthrax strains are dried in the same manner as the other cultures of which we have been speaking. In practice the institute uses 20 dried productive strains of the malignant anthrax pathogen.

Observation of dry cultures of malignant anthrax for 3 years showed that cultural, morphological, biochemical, antigenic, and virulent properties are completely retained. For instance, on meat-peptone-agar grow typical R-shaped colonies (Figure 28), while in meat-peptone-broth the cultures grow with characteristic clarification of the medium and the formation of clumps on the bottom of the test tube.

In a morphological sense the bacilli of malignant anthrax from a 24-hour broth culture made from dry strains are typical rods with slightly rounded ends (Figure 29) and the involution forms of microbes are lacking.

When tested for biochemical properties all strains of dry cultures of malignant anthrax manifest weak fermentative properties typical of the cultures, split up glucose, sucrose, and maltose with the formation of gas but do not change lactose.

Experiments to test the virulent properties of dry cultures of malignant anthrax over a period of 3 years showed that all the strains possessed great virulence. They caused death in rabbits inoculated in doses covered by current instructions within a period of 36, 48, 60, and 72 hours.

The use of dry strains of malignant anthrax for cultivation and the preparation of antigen in the hyperimmunization of horses which produce antianthrax serum has shown that the strains have pronounced antigenic properties. This fact can be confirmed by the fact that the antianthrax serum obtained possessed very active properties against malignant anthrax.

In addition to the microbial cultures described above the laboratories of the State Control Institute dry many other species of microorganisms, for example, cultures of pullorum disease, glanders, paratyphoid abortion of mares, etc.

A study of the physicochemical properties of dry cultures (pH of the medium, residual moisture, and viscosity) showed the following. The reaction of the medium in cultures following desiccation shifts somewhat toward alkalinity. The residual moisture in dry cultures does not exceed 0.4-0.7% when determined in a desiccator over calcium chloride under vacuum and 1.0-1.5% when tested in a drying cabinet at 105°. The viscosity of cultures as a result of desiccation undergoes almost no changes. We determined viscosity by Determan's method.

The results of our examinations in this area are provided in Table 23.

TABLE 23  
RESULTS OF TESTING pH, RESIDUAL MOISTURE,  
AND VISCOSITY OF DRY MICROBIAL

CULTURES		pH	Residual Moisture	Viscosity
Name of Culture	Culture Type			
Salmonella enteritidis	agar	7.3	-	1.6
Salmonella enteritidis	dry	7.7	0.6	1.7
Salmonella cholerae-suis	agar	7.4	-	1.5
Salmonella cholerae-suis	dry	7.7	0.45	1.5
Brucella suis	agar	7.4	-	1.6
Brucella suis	dry	7.6	0.73	1.8
Pasteurella suisepitica	broth	7.0	-	1.7
Pasteurella suisepitica	dry	7.4	0.52	1.6

In describing the method for drying microbial cultures used in the State Control Institute we must indicate the methods for drying microorganisms used in medical institutes. For instance, A. Klimentova, R. Kruk, and G. Yarmol'chuk dried bacterial cultures of diphtheria and Shiga and Flexner dysentery. The cultures were freeze-dried under vacuum with normal serum used as a protective medium. When the dry cultures were tested the authors found that diphtheria microbes retained cultural, biochemical, and virulent properties for 4 years while the microorganisms of Shiga and Flexner dysentery retained their cultural, toxigenic, and agglutininogenic properties for 6 years.

K. Ye. Dolinov and L. B. Balsyan dried pneumococcal cultures from a frozen state under vacuum. They used normal serum and culture broth as a protective medium. In describing the desiccation method the authors point out that the prefreezing temperature for pneumococcal cultures of between  $-20^{\circ}$  and  $-70^{\circ}$  has the same effect on the viability of microbes. For this reason the use of the temperature of  $-78^{\circ}$  for prefreezing is unnecessary since the temperature of  $-20^{\circ}$  assures equally successful desiccation of the microbes.

K. Ye. Dolinov and L. B. Balayan established the fact that a serum medium is much more effective since the viable properties of the pneumococci are better preserved with it. The viable properties of the microbes are likewise better preserved during storage under vacuum.

S. S. Rezhemskiy reported on the successful desiccation and storage of *Escherichia coli* and *Vibrio aquatilis*. The cultures were dried from a frozen state in a vacuum apparatus.

For freezing they used temperatures between  $-20^{\circ}$  and  $-75^{\circ}$ , but Rechmenskiy believes that  $-20^{\circ}$  would be enough since this cooling temperature assures the production of high grade dry cultures. The dry culture of *Vibrio aquatilis* is easily restored by transfer to agar plates but the activity of growth of the *Vibrio* and its mobility in the first generation was weaker than the *Vibrio* strain in the control culture. This difference soon disappeared in later generations. When studying *Escherichia coli* Rechmenskiy discovered that with regard to morphology, acid-formation from lactose, toxicity for rabbits, and agglutinative properties the dry culture behaved in a typical fashion. The effectiveness of the storage method and the use of microbial cultures in the dry form has now been confirmed by its use in many microbiological institutes of the Ministry of Public Health USSR (Institute of Epidemiology, Microbiology, and Infectious Diseases of the Academy of Medicine USSR, Central State Control Institute imeni Tarasevich, and a number of other institutes).

M. M. Paybich, using sucrose and agar in drying bacterial cultures, observed that sucrose increases the resistance of bacteria to freezing, desiccation and in part to storage while agar contributes to a longer preservation period for the cultural and biological properties of microbes in a dry state. The use of gelatin is connected only with the necessity for improving drying conditions.

We cannot however agree with this fully, since the dying process among microbial cells of many dry cultures prepared with the addition of sucrose and gelatin, in the experiments of Kozlov, Del'nik, and Vinogradov, was quite intense. The number of dead

microbial cells in many cultures during storage in a refrigerator (+2°, -4°) during the course of a year increased to extremely great dimensions. For instance, the number of viable microbes of *Escherichia coli* dropped to 8.3%, of paratyphoid A to 0.76%, of paratyphoid B to 3.1%, of brucellosis to 3.4%, Flexner's dysentery to 1%, and Sonne's dysentery to 0.7%. This suggests that the dying process in microbial cells of cultures dried with the addition of sucrose and gelatin had a very rapid course and this might be caused only by the insufficient richness of the drying medium, that is, sucrose and gelatin.

This fact is confirmed by our experiments and the experiments of Dolinov and Balayan where other results on the viability of microbes were received when normal serum was added as a protective medium for desiccation. For instance, in our experiments on drying *Salmonella enteritidis* with serum and in studying viability factors it was determined that after 3 years of preservation the number of viable cells was 30.4% and when dried with gelatine the culture no longer produced any growth when transferred to culture media. It must be pointed out that in our experiments the dry *Salmonella enteritidis* cultures were kept at ordinary room temperatures of 15°, 20°, and 27° while in Kozlov's, Del'nik's, and Vinogradov's experiments the dry cultures were stored at a more favorable temperature of +2°, -4°.

Analogous results were obtained by Dolinov and Balayan. Dry pneumococcal cultures, dried with serum, retained 33-79% viable cells after 38 months while in a pneumococcal culture dried with nutritive broth there remained only 2.8-8% viable cells after the same time.

G. V. Vygodchikov, in treating problems of preserving microbial cultures used for making vaccine preparations, points out that for this purpose a fully satisfactory method was that of drying microorganisms in a vacuum apparatus from a frozen state. The author believes that for a basic improvement in vaccines, used for the prevention of intestinal infections, we need to use the principles of controlled variability of microbes and the selection of the most immunogenic strains with subsequent preservation of their biological properties through desiccation.

In 1950 M. V. Pelevina's work on drying microbes at the Central Control Institute imeni Tarasevich was published. The author reports that desiccation took place at first in a desiccator without a vacuum and then in a Flosdorf vacuum apparatus, showing the superiority of drying the microbial cultures from a frozen state in a vacuum apparatus. In the first experiments M. V. Pelevina dried typhus and paratyphoid A and B cultures on filter paper which was impregnated with a culture washed from agar by normal horse serum and placed in ampules. A study of these cultures after 30 months storage in a dry state showed that they had not changed their biological properties.

In subsequent experiments M. V. Pelevina dried 65 strains of various microbial cultures including 25 strains of Shiga dysentery, 11 strains of Schmitz-Stutzer's dysentery, 6 strains of Sonne's dysentery, 2 strains of typhoid fever, 5 strains of *Escherichia coli*, 4 strains of *staphylococcus*, etc. For drying media M. V. Pelevina used normal horse serum, a galatine-agar culture with sucrose, and skimmed milk. Cultures of 0.2 ml in

ampules were dried in a manifold vacuum apparatus for 6 hours.

An additional 10-hour desiccation took place with the vacuum pump turned off. By determining the number of viable microbial cells in the dry cultures, M. V. Pelevina came to the conclusion that the gelatine-agar-sucrose medium had greater protective properties.

However, it must be pointed out that the percentage of dying microbial cells in all cultures including those with the gelatine-agar-sucrose medium was extremely high (99.5, 99, 95, 90, 85). The amount of residual moisture in the dry cultures was 5-11%. The data cited by Pelevina on the effectiveness of the serum medium for the desiccation of cultures are at great divergence with the data obtained by K. Ye. Dolinov and the author of the present book. Unfortunately, the observations of Pelevina with regard to the quantity of viable microbes in dry cultures, as shown in the article, were made only during the course of 6 months. It would be extremely interesting to carry out these observations over a longer period of time. As a result, Pelevina comes to the conclusion that the experiments conducted grant us the practical application of shipping strains of microbial cultures in a dry form.

While studying the effect of freezing and desiccation on the viability of microbial cultures of *Salmonella enteritidis*, we determined that a large number of microbial cells die during freezing. For instance, when using normal horse serum as a protective medium 40% of the microbes die, with gelatine 45% die, and with a physiological solution 87%. The dying of microbial cells continues even during desiccation and reaches 12, 11, and 6% for the 3 media, respectively.

We may assume that during the freezing process and during desiccation the weakest microbial cells die first. Later dying during storage in the dry state is much less pronounced and especially in cultures dried with normal serum. The number of dead microorganisms dried with serum during a 3-year storage period was only 33.2%. This shows the possibility for keeping microbes in a dry state for a long time.

On the basis of our investigations we can understand the absence of any data in the literature on the dying of microbial cells during culture desiccation. This apparently depends wholly on the external conditions under which freezing, desiccation, and storage take place.

We obtained better results in drying microbial cultures by using inactivated normal horse serum as a protective medium. For this reason we used this medium in all later observations.

A systematic 4-year study of all microbial cultures which we had dried and observations of them during a later period permitted us to determine the biological pattern of the condition of microbial cultures during desiccation and storage.

As we see from the preceding experiments in this section of the book, a protein protective medium and a high vacuum contribute to prolonged preservation of the viable properties of microbial cells in dry cultures. Microbial cultures retain fully their morphological, cultural, biochemical, agglutinative, toxic, virulent, and antigenic properties for no less than 4 years.

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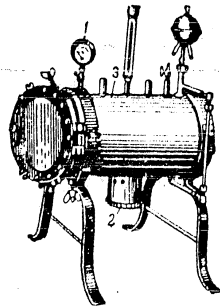


Figure 1. Vacuum anaerostat. 1, vacuum manometer; 2, attachment for heating; 3, water jacket.

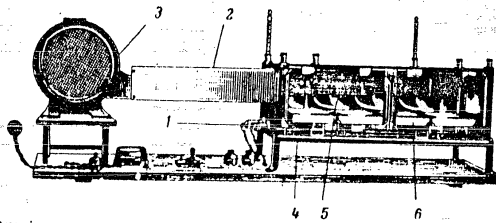


Figure 2. Drying apparatus with air and water heating. 1, heating element; 2, element for heating forced air; 3, fan for blowing in air; 4, hollow space for heated water between walls; 5, slots for access of forced air to material being dried; 6, shallow trays for material being dried.

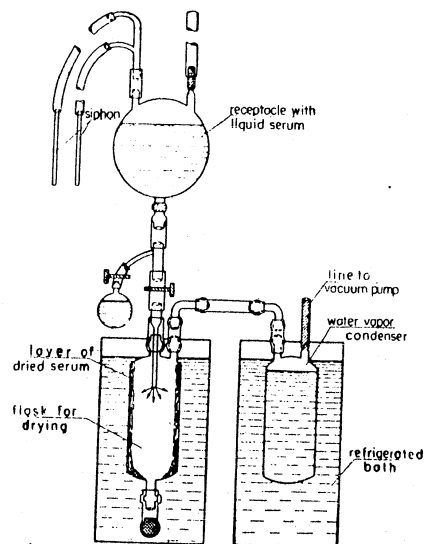


Figure 3. Spray-type vacuum apparatus (diagram).

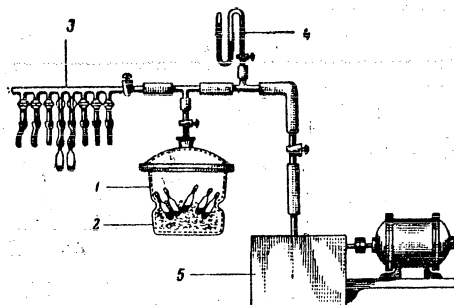


Figure 4. Vacuum drying device. 1, desiccator with culture; 2, chemical moisture absorbent; 3, connecting tubes for sealing ampules with cultures; 4, vacuum manometer; 5, vacuum pump.

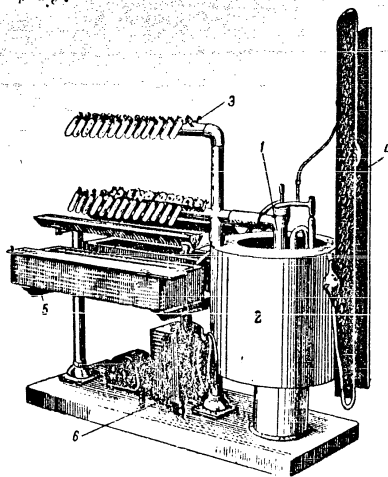


Figure 5. Manifold-type vacuum apparatus. 1, refrigerated condenser; 2, condenser bath; 3, manifold with ampules; 4, vacuum manometer; 5, shallow tray for freezing mixture and warm water; 6, oil vacuum pump.

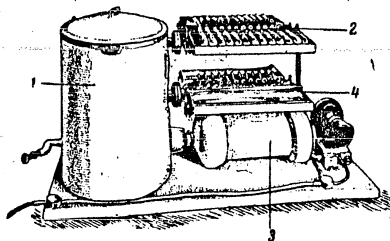


Figure 6. Manifold-type vacuum apparatus. 1, chamber for calcium sulfate; 2, manifold with ampules; 3, electric furnace for regeneration of calcium sulfate in chamber; 4, shallow tray for freezing liquid and warm water.

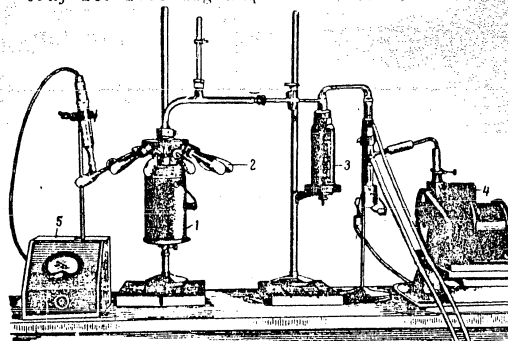


Figure 7. Radial manifold-type vacuum apparatus. 1, main condenser; 2, manifold with ampules; 3, supplementary condenser (trap); 4, oil vacuum pump; 5, vacuum apparatus.

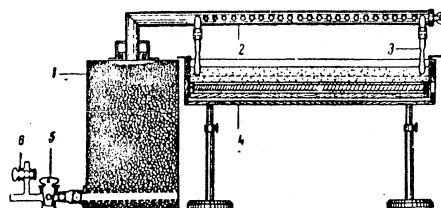


Figure 8. Manifold-type vacuum apparatus. 1, chamber with calcium sulfate; 2, manifold; 3, ampule; 4, shallow tray for freezing mixture and warm water; 5, stopcock in vacuum line; 6, stopcock in line to vacuum manometer.

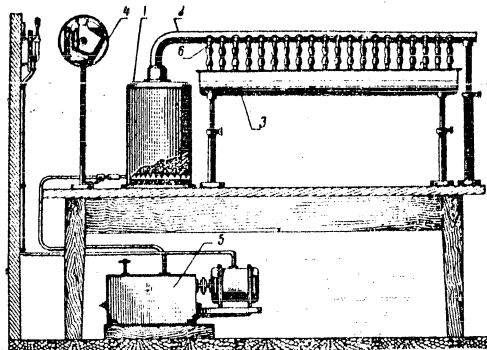


Figure 9. Manifold-type vacuum apparatus. 1, chamber for calcium sulfate; 2, manifold; 3, shallow tray for cooling mixture and warm water; 4, vacuum manometer; 5, oil vacuum pump; 6, connecting tubes with ampules.

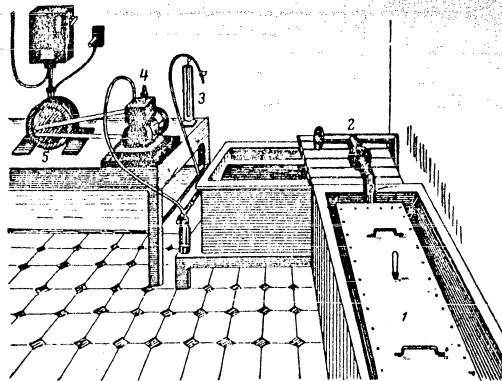


Figure 10. Chamber-type vacuum apparatus. 1, drying chamber; 2, condenser; 3, vacuum manometer; 4, oil vacuum pump; 5, electric motor.

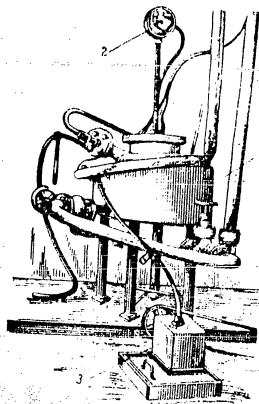


Figure 11. Chamber-type vacuum apparatus. 1, chamber for condensation and drying; 2, vacuum manometer; 3, oil vacuum pump.

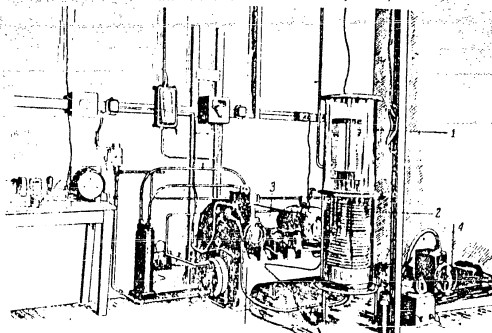


Figure 12. Chamber-type vacuum apparatus. 1, drying chamber; 2, condenser coil; 3, compressor installation; 4, oil vacuum pump.

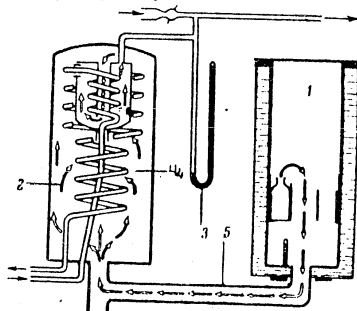


Figure 13. Chamber-type vacuum apparatus. 1, drying chamber; 2, condenser chamber; 3, vacuum manometer; 4, condenser coil; 5, connecting tube.

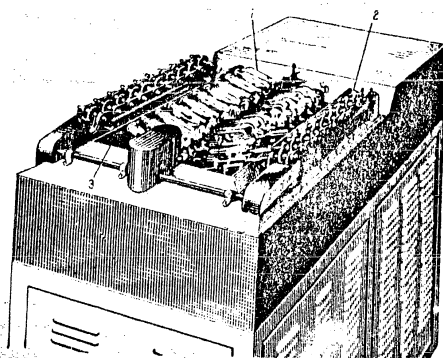


Figure 14. Freezing apparatus. 1, ampule with plasma; 2, rollers for turning ampules; 3, bath with freezing mixture.

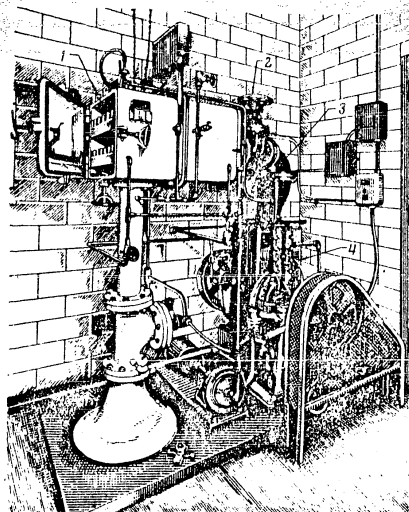


Figure 15. Cabinet-type vacuum chamber apparatus. 1, drying chamber; 2, closing valve; 3, pipe; 4, oil vacuum pump with centrifugal purifier.

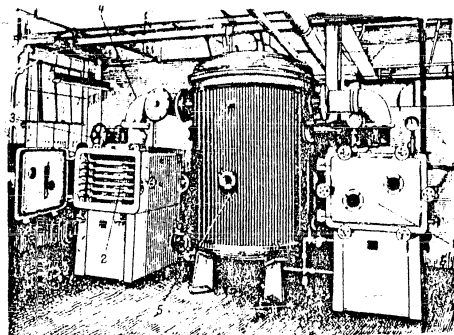


Figure 16. Cabinet-type vacuum chamber apparatus. 1, drying chamber; 2, chamber shelf for setting preparation; 3, closing valve; 4, pipe; 5, condensation chamber with coils.

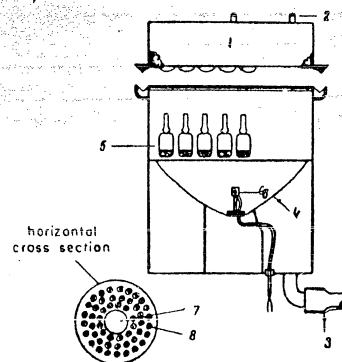


Figure 17. Chamber-type vacuum apparatus with infrared radiation (diagram). 1, refrigerator condenser; 2, cooling agent inlet; 3, line to vacuum pump; 4, parabolic reflector; 5, ampoules with preparation; 6, heating element in reflector focal point; 7, flat portion of reflector; 8, ampoules with preparation.

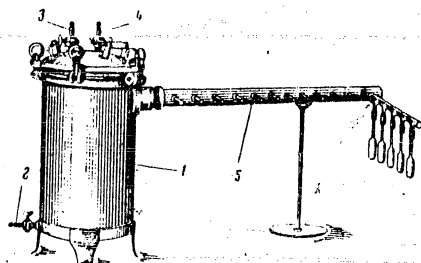


Figure 18. Chamber-manifold vacuum apparatus. 1, chamber containing preparation and calcium sulfate when chamber drying system is used and calcium sulfate only when manifold drying system is used; 2, vacuum line for pumping out air when manifold drying system is used; 3, vacuum line to manometer; 4, vacuum line for removing air when chamber drying system is used; 5, manifold for desiccation.

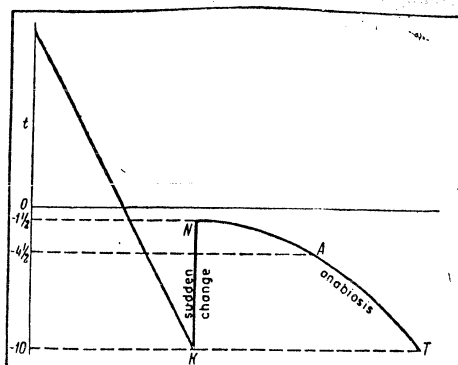


Figure 19. Temperature curve for an insect body during freezing (according to Bakhmet'yev). Explanation in text.

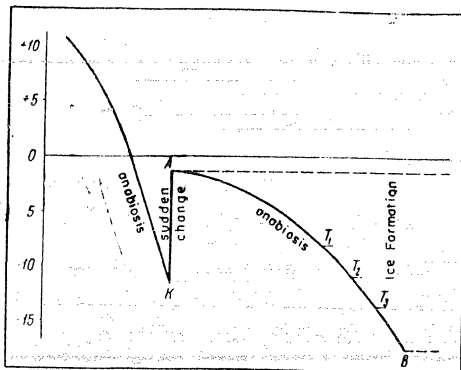


Figure 20. Temperature curve of freezing and anabiotic state of animals (according to Shmidt). Explanation in text.

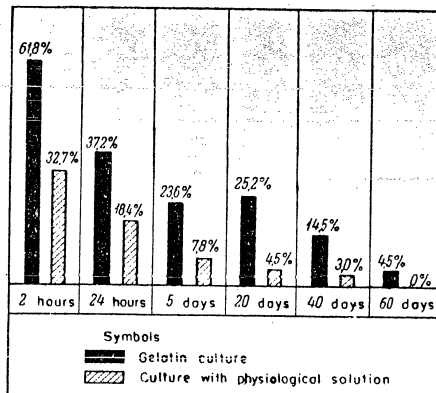


Figure 21. Action of low temperature ( $-17^{\circ}$ ,  $-20^{\circ}$ ) on the viability of microbe from a culture of *B. anthracis*. Explanation in text.

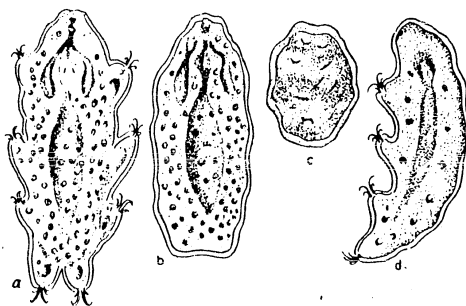


Figure 22. Tardigrade stained with neutral red. a, after 3 days in a neutral red solution; b, beginning of desiccation; c, in a dried state; d, after resuscitation (had been 36 days in dry state at  $15^{\circ}$ ).

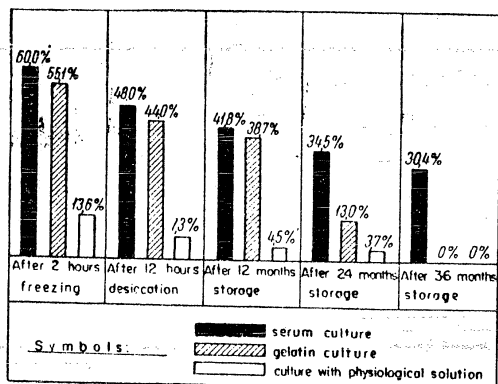


Figure 23. Viability of bacteria from a culture of calf paratyphoid pathogen during freezing, desiccation, and storage. Explanation in text.

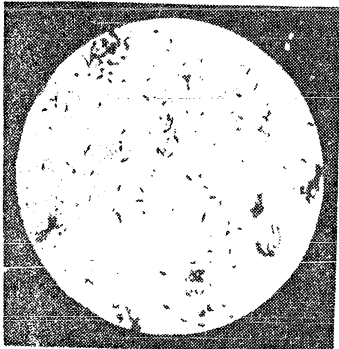


Figure 24. Microbes in a dry culture of the calf paratyphoid fever pathogen.

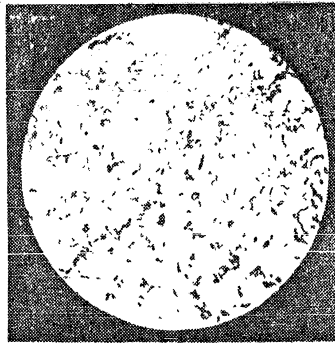


Figure 25. Microbes in a 24-hour agar culture of the pathogen of calf paratyphoid fever, cultured from a dry stain.

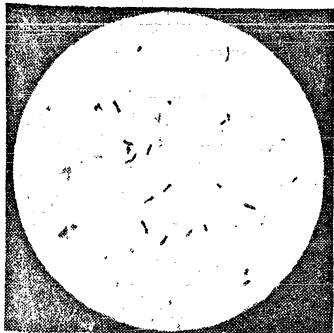


Figure 26. Microbes in a dry culture of the pathogen of anaerobic dysentery of lambs.

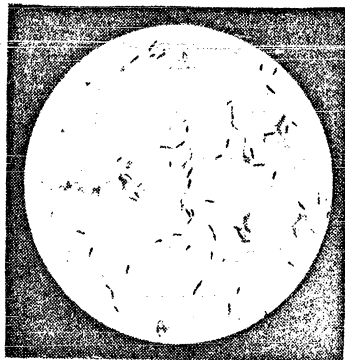


Figure 27. Microbes in a broth culture of the pathogen of anaerobic dysentery of lambs cultured from a dry stain. Explanation in text.

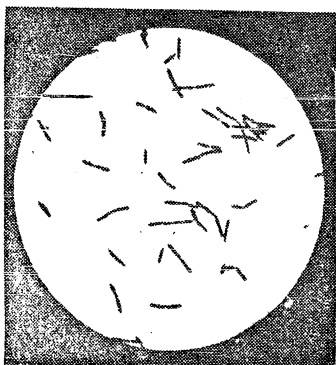


Figure 28. Colonies of a 24-hour agar culture of malignant anthrax, cultivated from a dry stain.

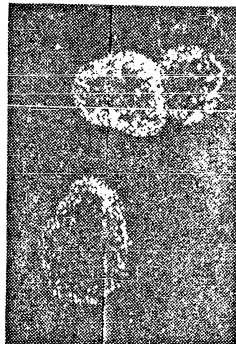


Figure 29. Microbes in a dry culture of the malignant anthrax pathogen.